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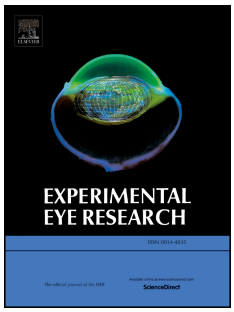

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Gut microbiota compositional profile and serum metabolic phenotype in patients with primary open-angle glaucoma

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Abstract

The gut microbiota (GM) and its influence on host metabolism are considered to be an environmental factor that contributes to the progression of many immune and neurodegenerative diseases. However, the features of the GM and serum metabolites in Primary open-angle glaucoma (POAG) patients have not been clearly elucidated. The purpose of this research is to explore the gut microbial composition and serum metabolic phenotype in POAG patients. 16S rRNA V4 genes of bacteria from the fecal samples of 30 POAG patients and 30 healthy subjects were sequenced by the Illumina MiSeq platform and then analyzed by QIIME. Their serum samples were analyzed by gas chromatography/mass spectrometry (GC-MS)-based metabolomics.

The association between gut microbial species and host circulating metabolites and clinical phenotypes was also analyzed. Compared with controls, *f_Prevotellaceae*, *g unidentified Enterobacteriaceae*, and *s_Escherichia coli* increased the most in POAG patients, whereas *g_Megamonas and s_Bacteroides plebeius* significantly decreased in POAG patients. The alteration of the endogenous metabolomic profile in POAG patients included five amino acids or dipeptides, two hormone derivates, one purine derivative, one bile acid derivative and one organic acid. It also showed that citric acid was positively correlated with *Megamonas*, whereas L-γ-Glutamyl-L-alanine, MHPG, cholic acid glucuronide and hypoxanthine were negatively correlated with *Megamonas*. Mean visual acuity was negatively correlated with *Blautia*, mean VF-MD was negatively correlated with *Faecalibacterium*, and average RNFL thickness was positively correlated with *Streptococcus*. Our results revealed that there was a distinct difference in GM composition and serum metabolic phenotype between POAG patients and healthy individuals. This finding suggests the potential correlations between the GM and serum metabolites in the pathogenesis of glaucoma and thus provides new insight into the GM-targeted interventions of this disease.

Keywords: primary angle-open glaucoma, gut microbiota, serum metabolites

Introduction

Glaucoma is the primary cause of irreversible blindness, which affects approximately 70 million people worldwide (H. Quigley & Broman, 2006). Primary open-angle glaucoma (POAG) is the main type of glaucoma, and information on established risk factors for the disease is limited. Intraocular pressure (IOP) is currently thought to be the most important risk factor and directly causes impairment to retinal ganglion cells (RGCs). However, RGCs and their axons also gradually deteriorate in individuals with glaucoma whose IOP is kept under a critical point, suggesting other potential mechanisms in the process of neurodegeneration. A number of studies have indicated that autoimmune factors, mitochondrial dysfunctions, environmental factors, and genetic factors may also lead to RGC damage in glaucoma (K. Abu-Amero, Kondkar, & Chalam, 2015; Kim & Varma, 2010; Rieck, 2013). It was indicated that a lack of induction of Treg cells and disproportionate pro-inflammatory and anti-inflammatory reaction patterns of Th cells existed in some normal-tension glaucoma (NTG) patients (Guo et al., 2018). In another study, elevated DNA mutations in mitochondria and a decrease in mitochondrial respiratory function in peripheral blood were also linked to POAG (K. K. Abu-Amero, Morales, & Bosley, 2006). Germ-free (GF) mice did not present glaucomatous heat shock protein and T-cell response or related glaucomatous neurodegeneration (Chen et al., 2018).

Interestingly, the human intestinal flora and its influence on the host metabolism have been shown to play a crucial role in many immune diseases, including, but not limited to, arthritis, Behcet's syndrome, ankylosing spondylitis and autoimmune uveitis (Berer et al., 2011; Consolandi et al., 2015; Gill, Asquith, Rosenbaum, & Colbert, 2015; Nakamura et al., 2016; Scher et al., 2013). The gut microbiota–immune axis may affect the immune balance in these diseases. In addition, in some neurodegenerative diseases, the microbiota-gut-brain axis has been well studied. It was extensively observed that there were significant microbiome differences, such as a large quantity of *Lachnospira and Akkermansia,* within patients who suffered from amyotrophic lateral sclerosis, Parkinson's disease, and multiple sclerosis (MS), while advantageous microbes such as bacteria that produce butyrate were extremely decreased in the aforementioned diseases (Hsiao et al., 2013; E. M. M. Quigley, 2017).

Given the similarities of immune and neurodegenerative factors between POAG and these diseases, we hypothesized that the gut microbiome and serum metabolites may also be correlated with the pathogenesis of glaucoma. To solve this problem, we applied 16s rRNA sequencing to analyze the microbiota of stool samples from 30 POAG patients and 30 healthy individuals, and we performed a metabolomic analysis of serum samples from the two groups. The association between the GM and the host serum metabolome and clinical phenotypes was also investigated to discover its potential pathogenic role in POAG.

Materials and methods

Subject recruitment and metadata collection

This project was approved by the medical ethics committee of Zhongshan Ophthalmic Center (No. 2019KYPJ100). All participants gave informed consent, and the study was performed in accordance with the tenets of the Declaration of Helsinki.

A total of 60 subjects were recruited for this study, including 30 POAG patients and 30 non-POAG healthy individuals who were age- (to within 5 years) and sex-matched, all undergoing a common physical examination. All participants were currently located in the city of Guangzhou and had the same ethnic background with similar eating habits. They were recruited for the current study between January 2019 and July 2019.

Eligibility for glaucoma patients required meeting the following clinical and visual field criteria: having open angles (at least Shaffer grade III), the existence of a typical glaucomatous visual field defect, and a typical cup/disc ratio (CDR) >0.6 in at least one eye. All included POAG patients had elevated IOP at the initial diagnosis, and patients with isolated ocular hypertension and any secondary form of glaucoma were excluded. The patients with glaucoma had no memory decline, altered mental status, limb tremors, tardive movements, muscle rigidity or other neurological symptoms. If there were suspicious symptoms, we asked the neurologist to rule out

neurodegenerative diseases. Patients with glaucoma did not have any other ocular diseases. Control group eligibility required meeting the following criteria: visual acuity ≥ 20/50, no current or past IOP elevation (IOP<21 mmHg), no prior use of IOP-lowering medications, a CDR difference < 0.2 and CDRs <0.4 in both eyes, the absence of any other intraocular pathology, and no family history of glaucoma. The enrollment of the two groups occurred at the time of the scheduled regular ophthalmic examination, which included measurements of best corrected visual acuity (BCVA), IOP, gonioscopy and fundus papillary morphology. In addition, glaucoma patients were subjected to automated visual field examination (HVF) and spectral optical coherence tomography (OCT) if they had not had such a test within 6 months of the time of enrollment. Exclusion criteria for the two groups included the following: a history of insulin, statin, corticosteroid, aspirin, nifedipine, metformin, and metoprolol use; a history of antibiotic or probiotic use within four weeks before enrollment; a history of diseases including, hypertension, diabetes, heart disease, renal failure, liver disease, chronic inflammatory bowel disease, psoriasis, chronic arthritis and cancer; a history of gastrointestinal tract surgery; or a history of prolonged diarrhea or chronic constipation.

A standardized self-reported questionnaire was designed specifically for this study, which covered participants' sociodemographic features, anthropometric information (BMI, SBP), information related to lifestyle (smoking, sleep time, current alcohol consumption, eating habits) and ocular medication history. All

information provided during the face-to-face interviews was recorded by the same investigator.

Fecal and serum sample collection

The individuals had not received any antibiotic treatment and had not consumed any food containing probiotics for at least one month before sample collection. A sterile stool sampler and a concomitant instruction about sample collection and storage were distributed to each participant. Most of the stool samples were collected in the morning and kept at 4 °C for no more than 6 hours and then quickly transferred to the laboratory and stored at −80 °C until use. Total genomic DNA from samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

Fasting blood samples from each participant were collected by qualified nurses and anticoagulated with EDTA-K2. At the laboratory, all blood samples were centrifuged for serum extraction. Each obtained 100 μL serum sample was mixed with 400 μL methanol and centrifuged at 4 °C, 12,000 rpm, for 15 min. Approximately 200 μL of the supernatant was collected and transferred into a new Eppendorf tube and dried using a vacuum concentrator. Then, all samples were redissolved in 60% methanol and analyzed by GC-MS.

16S rRNA Sequencing, Classification and Diversity Analysis

The 16S rRNA gene extracted from fecal samples was amplified following the standard procedures of the Earth Microbiome Project using a set of updated universal primers 515F/806RB specifically targeting the hypervariable V4 region. The V4 region was sequenced by the Illumina MiSeq (San Diego, CA, USA) and then processed through the workflow package Quantitative Insights Into Microbial Ecology (QIIME). The filtered sequences were clustered and grouped as operational taxonomic units (OTUs). Species alpha-diversity (Shannon index) was calculated to analyze diversity and richness within a community. Beta-diversity was calculated for the distance between communities. Finally, linear discriminant analysis effect size (LEfSe) analysis was applied to distinguish the differentially abundant taxa between two groups at the genus and phylum level (http://huttenhower.sph.harvard.edu/lefse/).

Metabolomic Analyses

GC-MS analysis was performed using a Vanquish UHPLC system (Thermo Fisher, Waltham, USA) with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher, Waltham, USA). Each sample was injected into a Hyperil Gold capillary column (100x2.1 mm, 1.9 µm thickness) at 320 °C with a flow rate of 0.3 mL/min. Eluent A (0.1% FA in Water) and eluent B (methanol) exhibited positive polarity, and eluent A (5 mM ammonium acetate, pH 9.0) with eluent B (methanol) exhibited negative polarity. The raw data files generated by the UHP GC-MS were

imported into Compound Discoverer 3.0 (Thermo Fisher, Waltham, USA) for calculating peak picking and peak alignment and for the quantitation of metabolites. The molecular formulas were predicted based on additive ions, molecular ion peaks and fragment ions. The accurate and relative qualitative results were obtained by matching peaks with Mzcloud (http://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/).

Statistical analysis

Statistical analysis was performed by SPSS 12.0. A bilateral Student's t-test was used to evaluate the group differences in the clinical data, and $p \leq 0.05$ was considered statistically significant. QIIME (Version 1.7.0) was used to calculate the Shannon index at the genus level. A two-tailed Wilcoxon rank-sum test was used to calculate the differential abundance of phyla, genera, and species in patients with POAG and healthy individuals. FactoMineR pack in R software (Version 2.15.3) was used to analyze the principal component analysis (PCA). A nonparametric Mann−Whitney-Wilcoxon test was used to analyze metabolomic data. The Benjamini−Hochberg correction method was performed for the comparison of multiple metabolite concentrations. Differentially enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified based on the Z-scores of the POAG group. The Cor.test function of the R statistical package was used to analyze Spearman's correlation between the GM and the serum metabolites and clinical

phenotypes (only the top 10 genera and top 20 metabolites and five clinical phenotypes [mean visual acuity, mean IOP at initial diagnosis, mean IOP, mean VF-MD, and average RNFL thickness]were performed; rho≥0.3, p≤0.05).

Results

Study population

A comparison of the demographic and ophthalmic data of the thirty POAG patients and thirty controls is presented in Table 1; their mean (± SD) age was 54.77 ±9.32 and 53.80±7.87 years, respectively. There was no difference regarding SBP, BMI, smoking, sleep time, or current alcohol consumption between the two groups. Ophthalmological examinations revealed that the POAG group had significantly lower visual acuity than the controls (P<0.01). No significant difference in IOP was found between the POAG group and the control group. Other general ophthalmic features (IOP at initial diagnosis for POAG patients, VF-MD, RNFL thickness, glaucoma medication) of the POAG patients are shown in Table 1.

Taxonomic assignment for collected samples and GM diversity in POAG patients and healthy subjects

We obtained a total of 4,404,345 high-quality clean reads from 30 POAG participants and 30 healthy subjects. A total of 1488 OTUs were clustered by reads at 97% identity. According to the OTU abundance and species annotation results, the

representative sequences of the top 100 genera were obtained by multiple sequence alignment, shown in evolution trees, and were mostly enriched in four phyla, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Figure 1). We constructed rarefaction curves and rank abundance curves to describe the sample diversity within the group. The rarefaction curves in the control and POAG groups suggested similar levels of gene richness in the two groups (Figure 2a). The rank abundance curve represented the richness and evenness of the species for the samples. The two groups showed an almost similar evenness of species (Figure 2b). Although the Shannon index (α-diversity) showed no significant difference between the two groups ($p = 0.8534$; Figure 2c), bacterial diversity in POAG subjects was slightly higher than that in healthy participants ($p=0.034$; Figure 2d).

Difference in the dominant microbiota between POAG patients and healthy control subjects

To analyze the difference in the dominant bacterial distribution between the POAG subjects and healthy individuals, LEfSe analysis and linear discriminant analysis (LDA) scores were used. LEfSe analysis is mainly used for the discovery and identification of two or more biological high-dimensional biomarkers and features of the genome, such as genes, metabolic pathways and classifications. LEfSe analysis uses the Kruskal-Wallis test and paired Wilcoxon rank-sum test to detect significant differences in the abundance and characteristics of the groups. Finally, LEfSe uses

LDA to estimate the influence of the abundance of each component (species) on the effect of the difference. A species with an LDA score greater than the set threshold (less strict is 2.0; more strict is 4.0) is the statistical biomarker. In our study, an LDA score >4 was considered a significant result. At the family level, *Prevotellaceae* was overrepresented in the POAG group. At the genus level, *unidentified_Enterobacteriaceae* was predominant in the POAG group, and *Megamonas* was more prevalent in the healthy subjects. At the species level, *Escherichia_coli* was overrepresented in the POAG group, whereas *Bacteroides_plebeius* was predominant in the control group (Figure 3a). The cladogram was also obtained using the LEfSe analysis. In POAG patients, the phylum *Prevotellaceae* showed a large effect size (Figure 3b).

Metabolic profiling of serum in POAG patients and healthy subjects

A total of 750 metabolites in positive mode (ES+) and 587 metabolites in negative mode (ES-) were finally identified. The principal component analysis (PCA) score plot was used to reveal the metabolic alteration types in the two groups. Samples from the two groups presented a scattered, dispersed plotting pattern, indicating a dramatic difference in the composition of metabolites between the POAG group and the control group (Figure 4a). To identify differential metabolites, we used the variable importance in the projection (VIP) value of the first principal component of the partial least squares discrimination analysis (PLS-DA) model and the P value of

the t-test. In the PLS-DA model, we obtained results of R2=0.92 and Q2=-0.52, implying predictability and reliability of this model (Figure 4b). A total of 35 metabolites were identified to differ in abundance between POAG patients and healthy subjects. Among these, 20 metabolites were increased significantly, and 15 metabolites were decreased obviously in POAG patients, and the variation tendencies were shown by a volcano plot (Figure 4c). Moreover, 10 endogenous metabolites were structurally identified, including five amino acids or dipeptides, two hormone derivates, one purine derivative, one bile acid derivative, and one organic acid (Table 2). KEGG analysis was also used to identify the most crucial signal transduction pathway and biochemical metabolic pathway relevant to differential metabolites. There were twenty KEGG modules with differential enrichments, and the largest number of metabolites was enriched in the pathway of microbial metabolism in diverse environments (Figure 4d).

Correlation of gut microbial species and host circulating metabolites and clinical phenotypes

The correlation of GM components and the serum metabolites and clinical phenotypes in POAG participants were investigated by Spearman's correlation analyses. The results revealed that citric acid was positively correlated with *Megamonas*. L-γ-Glutamyl-L-alanine, MHPG (3-methoxy-4-hydroxyphenylglycol) and hypoxanthine were negatively correlated with *Megamonas*. Citric acid was

negatively correlated with *unidentified_Enterobacteriaceae* (Table 2 and Figure 5). Mean visual acuity was negatively correlated with *Blautia* (rho=-0.389，p=0.034). Mean VF-MD was negatively correlated with *Faecalibacterium* (rho=-0.387, p=0.035). The average RNFL thickness was positively correlated with *Streptococcus* (rho=0.383，p=0.037).

Discussion

To date, only a few studies have focused on the association between intestinal flora and glaucoma (Astafurov et al., 2014; Chen et al., 2018; D. et al., 2017; Hayashi et al., 2012). Several large gaps in the knowledge of the GM and glaucoma, such as the gut microbial components and their influence on the serum metabolite profiles of glaucoma patients and the microbial and metabolic biomarkers for glaucoma detection and treatment, remain rarely explored. To fill these gaps, we performed a strategy based on 16s rRNA sequences and metabolomic analysis. We uncovered a significant diversity of gut microbial communities and bacterial populations between POAG patients and healthy individuals, as well as a distinct alteration of serum metabolites. Furthermore, the close correlations between certain altered metabolites and the GM have strengthened, to some degree, the evidence of the association between intestinal flora and POAG.

In this study, we *first uncovered a s*ignificant β-diversity in the gut microbial communities of POAG patients and healthy individuals, among which *Prevotellaceae,*

Escherichia_coli and an *unidentified Enterobacteriaceae* were most dramatically increased in the POAG group, whereas *Megamonas* and *Bacteroides plebeius* were decreased most obviously compared with the control group. Previous studies suggested that the intestinal bacterium *Prevotella* exacerbated epithelial inflammation in a colitis mouse model and thrived in a pro-inflammatory environment of rheumatoid and hypertension pathology (Balakrishnan, Luckey, & Taneja, 2019; Karbach et al., 2016; Scher et al., 2013). As one of the fermenting bacteria, *Prevotellaceae* can produce butyrate, which has been proven to be a strongly specific agent for Treg cell differentiation (Furusawa et al., 2013). These studies and our results indicate that *Prevotella* might also be linked to neuronal inflammation and immune damage in glaucoma. In addition, *Escherichia_coli*, as a common Gram-negative bacterium, showed a significant increase in the POAG group compared to the control group in our study. Gram-negative bacteria may elicit strong immune responses and promote pro-inflammatory cytokine, nitric oxide and eicosanoid secretion via its main product, lipopolysaccharide (LPS) (Lamping et al., 1998). Using two kinds of glaucoma mouse models, one study revealed that LPS can activate Toll-like receptor 4 (TLR4) and its downstream molecules, which are relevant to retinal local inflammation and complement activation (Astafurov et al., 2014). Thus, this result indicates that an increase in the *Escherichia_coli* population may also participate in the pathogenesis of POAG. Moreover, our study showed a reduction in gut *Megamonas* in POAG patients, which has also been reported in

several other diseases, such as chronic kidney disease (Lun et al., 2019). It seems that *Megamonas* may play a protective role in this disease, but whether it has such a role in glaucoma is unclear. Rare studies referred to gut microbes of *Bacteroides plebeius*; thus, the explanation of why it decreased dramatically in our study needs further investigation.

It has been proven that host circulating metabolites are relevant to gut microbial components and may participate in the pathogenesis of several diseases, such as hypertension, obesity and Crohn's disease (Haghikia et al., 2015; Jansson et al., 2009; Li et al., 2017). In our study, we found alterations in 10 endogenous metabolites in POAG patients and healthy individuals. Compared to healthy individuals, the metabolomic composition of POAG patients included five amino acids and amino acid dipeptides, two hormone derivates, one purine derivative, one bile acid derivative, and one organic acid. It has been reported that the amino acid levels in the aqueous humor of POAG patients and glaucoma model mice are higher than those in controls (Buisset et al., 2019; Schuettauf et al., 2007). Branched-chain amino acids (BCAAs) can stimulate ATP production and have been verified to play a role in attenuating photoreceptor cell apoptosis in the RP mouse model (Hasegawa et al., 2018). In our study, we found similar results: L-γ-Glutamyl-L-alanine was upregulated and Gly-l-pro and glycine were downregulated in the POAG group. In addition, 3a,7a-dihydroxycholanoic acid, which is a bile acid, was higher in POAG patients than in controls in our study. A previous study demonstrated that bile acids

may have a role in oxidative stress and apoptosis (Orellana, 2002). Therefore, these imbalanced metabolites might be linked to RGC impairment in glaucoma.

Interestingly, there was a positive correlation between decreased citric acid and *Megamonas*, which belongs to the phylum Firmicutes. It was proven that the citric acid cycle plays a pivotal role in regulating energy homeostasis and cell metabolism, which is correlated with the mitochondrial dysfunction of glaucoma (He et al., 2004). Several studies have shown that plasma citrate concentration might be a glaucoma biomarker in pediatric and adult populations (Fraenkl et al., 2011; Michalczuk, Tadeusz, Urban, Anna, & Bakunowicz- Łazarczyk, 2017). In addition, our study found that increased hypoxanthine was negatively correlated with *Megamonas* in the serum of POAG patients. It was proven that an increase in hypoxanthine was an indication of increasing de novo purine synthesis in optic atrophy-related disorders (Bocca et al., 2018). This interesting finding might indicate that the damage of RGCs might result from the participation of the altered GM that might exert a regulatory function on metabolites. 3-Methoxy-4-hydroxyphenylglycol (MHPG), which increased dramatically in POAG patients compared to controls in our study, showed a strongly negative correlation with *Megamonas*. A previous study showed a positive correlation between the salivary MHPG level and the severity of depression (Hamer, Tanaka, Okamura, Tsuda, & Steptoe, 2007). However, among our recruited POAG patients, 83.16% had a medication history of beta-blocker use (Table 1), which may also contribute to the increased serum level of MHPG (Kaiserman, Kaiserman,

Elhayany, & Vinker, 2006). More studies should proceed to explore further evidence of this issue and the role of other kinds of metabolite alterations in POAG patients.

The principal limitations of this research are the small-scale recruitment, all from the same city and a single hospital, and only a few metabolites were investigated. Thus, larger-scale prospective studies in the future are necessary to support our study results and to obtain results that could more fully reflect the changes in POAG or other types of glaucoma. Another limitation is that the serum was procured from the peripheral blood of the subjects and the metabolites seen therein could be result of multiple conditions. Therefore, it is difficult to distinguish serum metabolite changes due to the disease itself from those due to exogenous substances. We think this difficulty can be addressed by studying a larger group and performing targeted experiments on specific metabolites in the future. In addition, the gut microbiome may vary at different time points and physical conditions. The repeatability of the microbiome results can be demonstrated through obtaining different samples from the same individual or at different time points.

To our knowledge, this is the first study that focused on the GM profile and its association with serum metabolites in POAG patients. Certain bacteria might provide an environment or affect host metabolism that are related to the pathogenesis of glaucoma. Each gut microbe we found to be significantly altered needs to be studied by additional experiments, and GM modulation may be considered a part of the treatment of POAG.

Conflict of interest

All authors claimed there were no conflicts of interest.

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Figure legends:

Figure 1: Phylogenetic tree at the genus level. The color of the branches and sectors represents their corresponding gate, and the stacked column diagram outside the fan ring represents the abundance distribution information of the genera in different samples.

Figure 2: GM diversity in POAG patients and healthy subjects. (**a**) Rarefaction curves display the number of operational taxonomic units (i.e., clusters of sequences with >97% similarity in this case) detected based on the sampling intensity of the libraries. (**b**) Rank abundance curves reflect the richness and evenness of the species in the samples. (**c-d**) α-Diversity (Shannon index) and β-diversity based on the genus profile in the two groups.

Figure 3: Difference in the bacterial distribution between the POAG patients and the control subjects. **(a)** Comparative analysis of the microbial communities at the family, genus and species levels using the LEfSe method. LDA scores (log10) for the most prevalent taxa in POAG patients are represented on the positive scale, whereas negative LDA scores indicate enriched taxa in the controls. **(b)** Cladograms of six different taxonomic levels (from phylum to genus). Green circles and shadings show the significantly enriched bacterial taxa obtained in POAG patients. Red circles and shadings show significantly enriched bacterial taxa obtained in healthy controls.

Figure 4: Metabolic profiling of serum in POAG patients and healthy subjects. **(a)** PCA score plots of serum samples from POAG patients (blue circle) and healthy controls (orange square). **(b)** Validation of the OPLS-DA model (using 200 random permutations). **(c)** The volcano plot of differential metabolites. Grey represents a nonsignificant difference, red represents an increase in metabolites, and green represents a decrease in metabolites. (**d**) Bubble diagram of the KEGG pathway (only the top 20 are shown, red arrow indicates the pathway with the largest number of metabolites). The x-coordinate is the ratio of the number of differential metabolites in the corresponding pathway to the total number of identified metabolites. The size of

the point represents the number of differential metabolites in the corresponding pathway.

Figure 5: The correlation between the GM and serum metabolites. The correlation heatmap of the top 4 endogenous differential metabolites with the top 10 differential species at the genus level. The right side is the correlation coefficient, blue indicates positive correlation and red indicates negative correlation.

Table 1 Demographics and other characteristics of participants

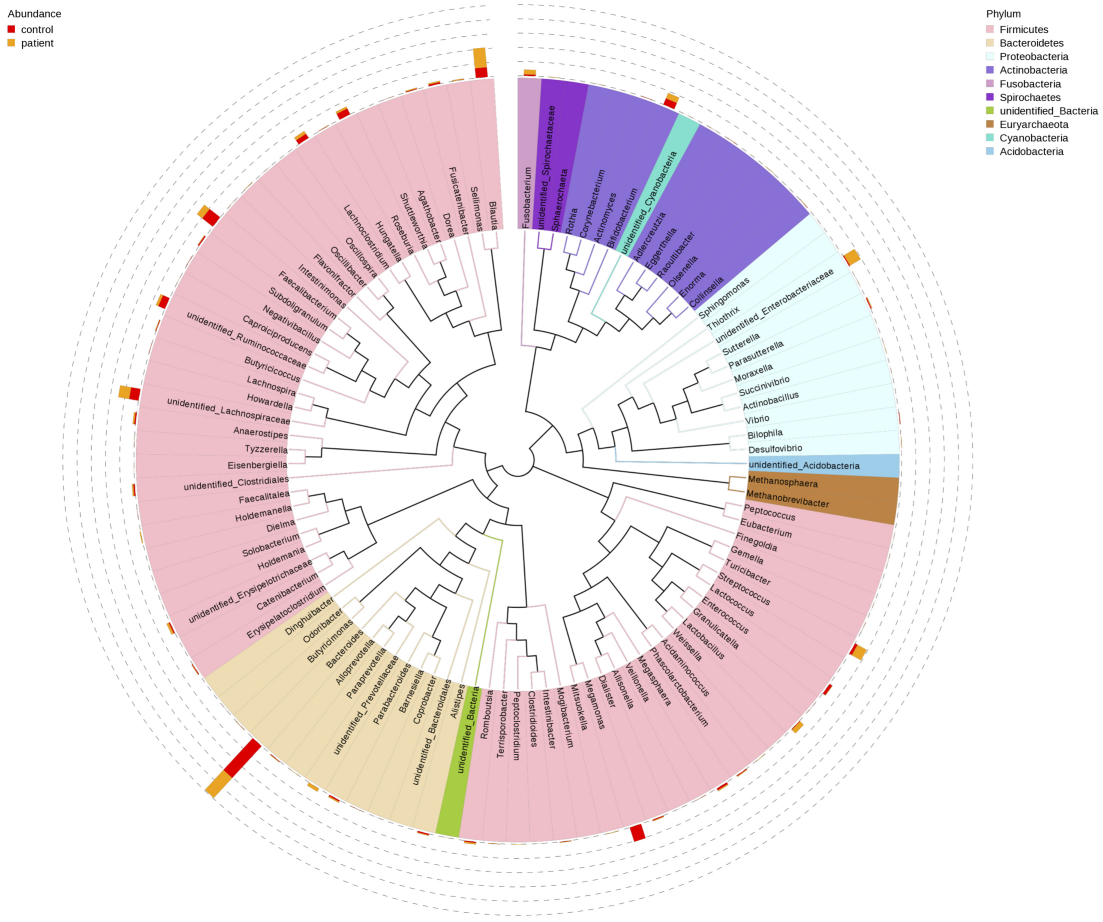
	POAG patients	Controls	P-values
Demographics and systemic comorbidity			
Gender: male/female	14/16	14/16	1
Mean age (years)	54.77 ±9.32	53.80±7.87	*NS*
Mean SBP (mmHg)	121.46±13.55	122.80±13.59	*NS*
Mean BMI (kg/m^2)	24.11±3.55	23.99±3.71	*NS*
Current alcohol consumption (mg/day)	35.00±65.85	33.33±60.64	*NS*
Smoking (per day)	2.26±4.63	2.20±4.56	*NS*
Sleep time (hours/day)	7.00±1.08	6.90±1.09	*NS*
Ophthalmological features and glaucoma medication			
Mean visual acuity, LogMAR	+0.12	+0.06	<0.01
Mean IOP at initial diagnosis	23.73±4.07		
Mean IOP (mmHg)	15.76±2.56	16.34±3.76	*NS*
Mean VF-MD (dB), worse eye	-7.62±5.12	-	
Average RNFL thickness (μm)	65.27±4.56	-	
Glaucoma medications (%)			
- Beta-blockers	83.16	-	
- Prostaglandin analog	45.15	-	
- Alpha-2-agonists	22.53	-	
- Carbonic anhydrase inhibitor	12.61	-	

SBP: systolic blood pressure; BMI: body mass index (weight/height^2); IOP: intraocular pressure; VF-MD: visual field mean defect. RNFL: retinal nerve fiber layer. *NS:* no significant difference (P>0.05).

Metabolite	VIP	P value	Up/Down	Associations	
				unidentified_ Enterobacteriaceae	*Megamonas*
Glycine	1.81	5.62E-13	Down		
Gly-l-pro	1.42	3.73E-06	Down		
Citric acid	2.35	7.53E-08	Down	**(-)**	**(+)**
Aspartyl-L-proline	1.65	1.24E-05	Down		
DL-Lysine	2.10	2.65E-13	Up		
L-γ-Glutamyl-L-alanine	3.61	1.36E-13	Up		**(-)**
MHPG	3.00	3.43E-06	Up		**(-)**
17-Hydroxypregnenolone sulfate	1.78	0.00306738	Up		
Hypoxanthine	1.85	0.02436963	Up		**(-)**
3a,7a-Dihydroxycholanoic acid	2.24	0.02610926	Up		

Table 2 Endogenous serum metabolites in POAG patients compared with controls

VIP: variable importance in the projection, P values were determined using Student's t-test. "+" represents positive correlation, "-" represents negative correlation.


Abundance
control
patient
Phylum
Firmicutes
Bacteroidetes
Proteobacteria
Actinobacteria
Fusobacteria
Spirochaetes
unidentified_Bacteria
Euryarchaeota
Cyanobacteria
Acidobacteria
Fusobacterium
unidentified_Spirochaetaceae
Spirochaeta
Rothia
Corynebacterium
Actinomyces
Bifidobacterium
unidentified_Cyanobacteria
Eikenella
Eggerthella
Rothia
Dialister
Erwinia
Gallimella
Sphingomonas
Thiothrix
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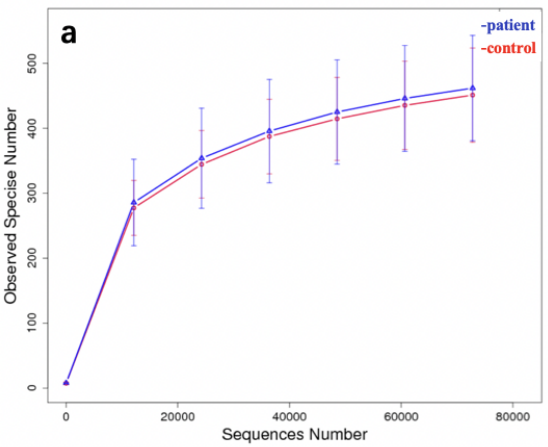
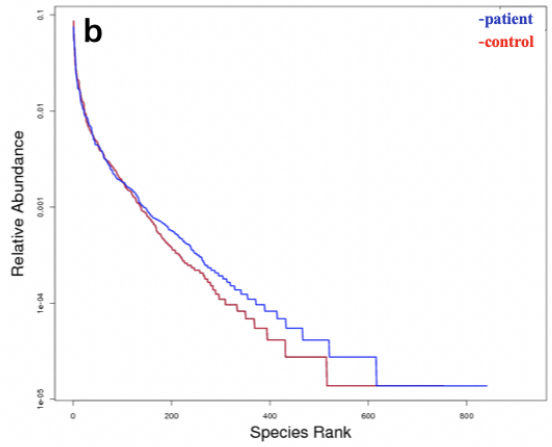
Sutterella
Parasutterella
Moraxella
Succinivibrio
Actinobacillus
Vibrio
Bilophila
Desulfovibrio
unidentified_Acidobacteria
Methanosphaera
Methanobrevibacter
Peptococcus
Eubacterium
Pinegoldia
Gemella
Turicibacter
Streptococcus
Lactococcus
Enterococcus
Granulicatella
Lactobacillus
Weissella
Anaerococcus
Finegoldia
Megasphaera
Veillonella
Allisonella
Dialister
Megamonas
Mitsuokella
Negativibacillus
Intestinibacter
Peptoclostridium
Clostridioides
Terrisporobacter
Romboutsia
unidentified_Bacteria
Alistipes
unidentified_Bacteroidales
Coprobacter
Barnesiella
Parabacteroides
unidentified_Prevotellaceae
Paraprevotella
Alloprevotella
Bacteroides
Butyricimonas
Odoribacter
Dingchunbaoa
Erysipelatoclostridium
unidentified_Erysipelotrichaceae
Catenibacterium
Holdemania
Solobacterium
Dielma
Holdemanella
Faecalitalea
unidentified_Clostridiales
Eisenbergiella
Tyzzerella
Anaerostipes
unidentified_Lachnospiraceae
Howardella
Lachnospira
Butyricicoccus
unidentified_Ruminococcaceae
Capraciproducens
Negativibacillus
Subdoligranulum
Faecalibacterium
Intestinimonas
Flavonifractor
Oscillibacter
Oscillospira
Lachnoclostridium
Hungatella
Roseburia
Shuttleworthia
Agathobacter
Dorea
Fusicatenibacter
Sellimonas
Blautia


a
-patient
-control
Observed Specise Number
Sequences Number
0
100
200
300
400
500
0
20000
40000
60000
80000


b
-patient
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Relative Abundance
Species Rank
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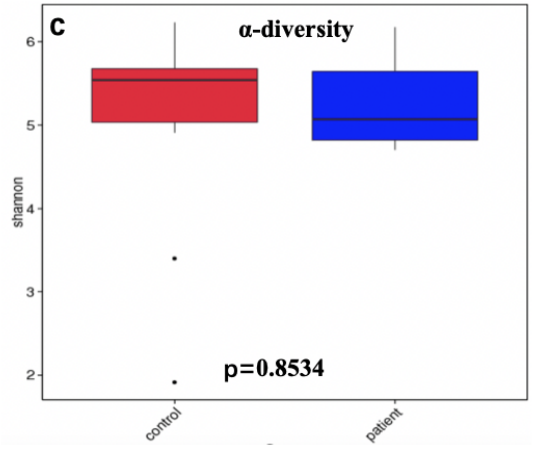
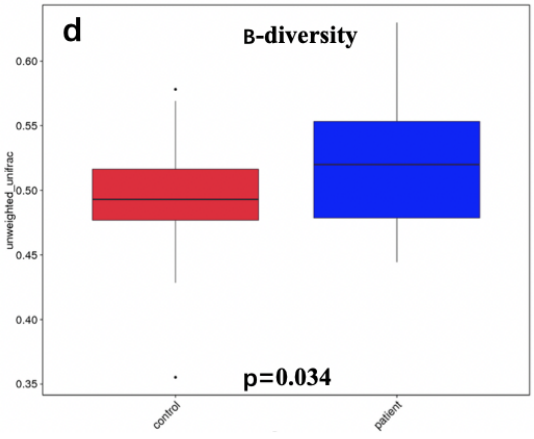
0.01
0.001
1e-04
1e-05
0
200
400
600
800


c
α-diversity
shannon
p=0.8534
control
patient
2
3
4
5
6


d
B-diversity
unweighted_unifrac
p=0.034
control
patient
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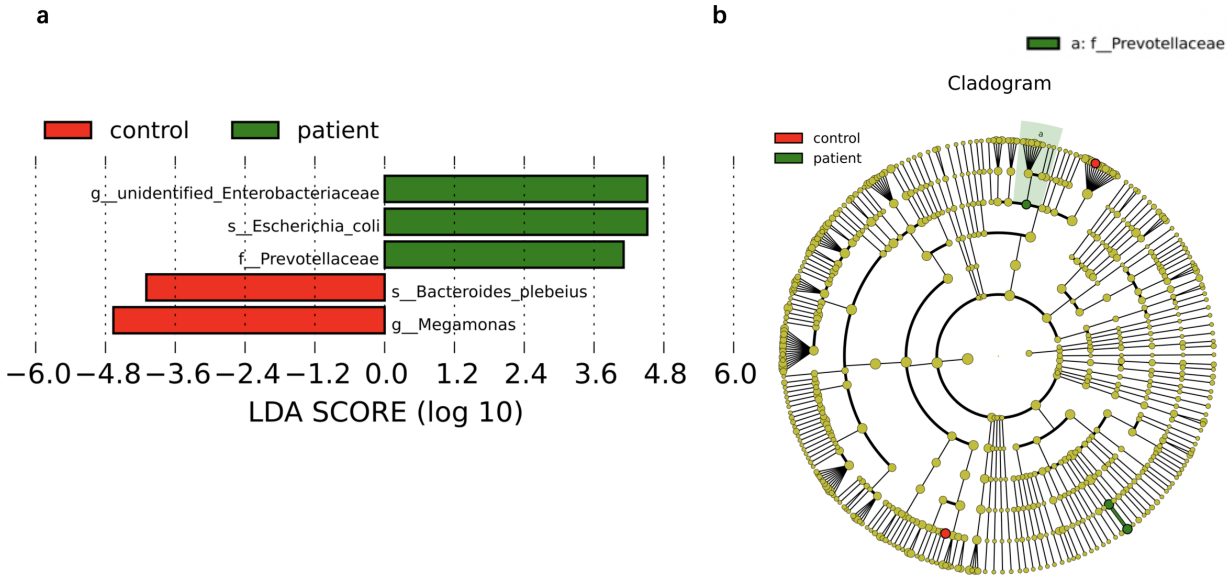
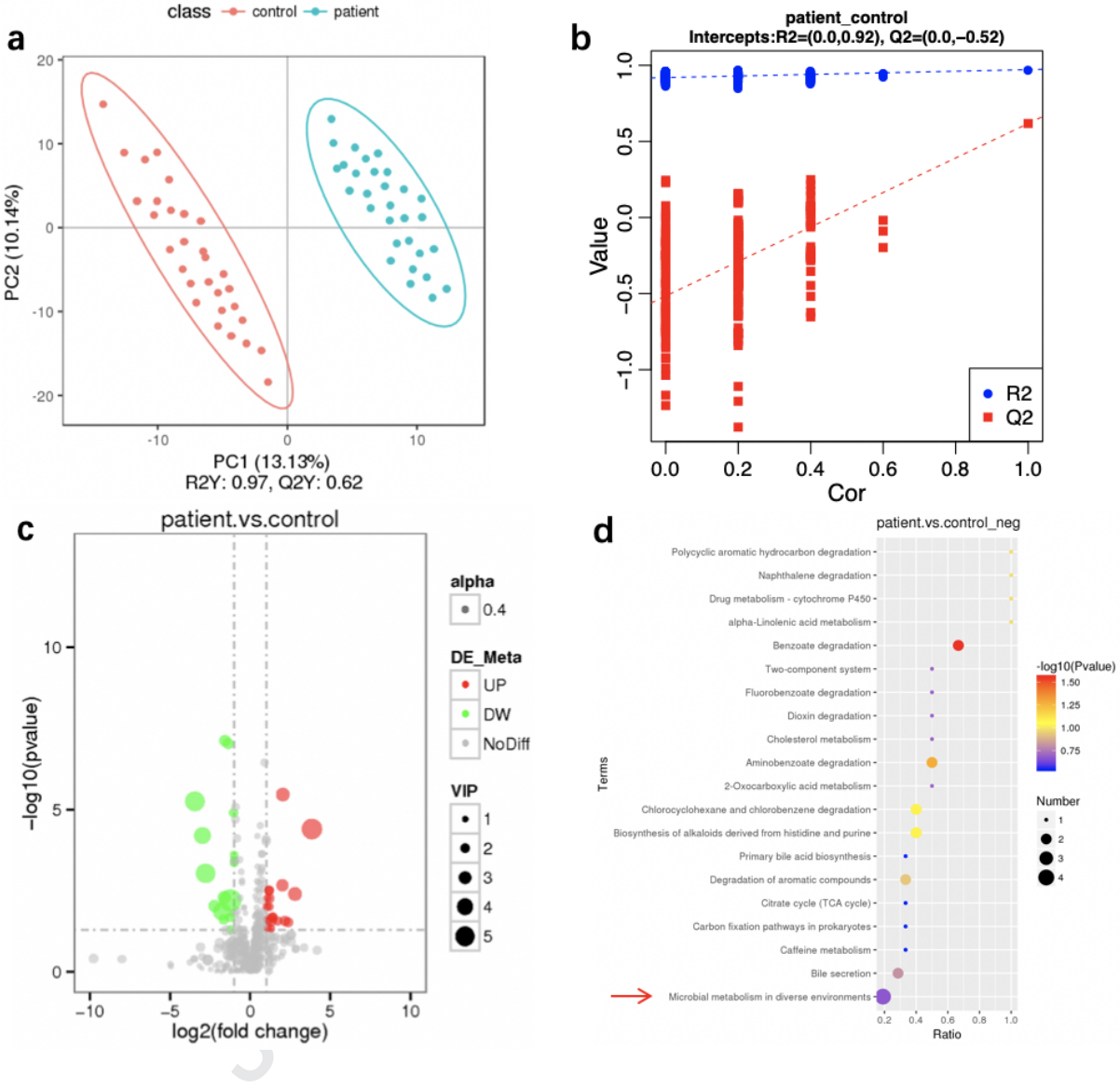
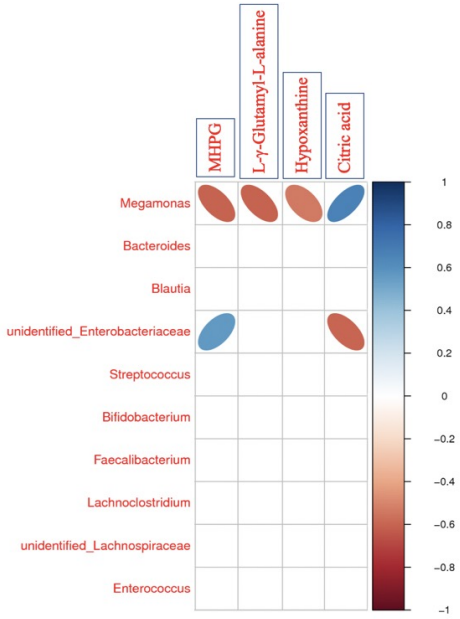
0.40
0.45
0.50
0.55
0.60




a
class control patient
PC2 (10.14%)
PC1 (13.13%)
R2Y: 0.97, Q2Y: 0.62
b
patient_control
Intercepts:R2=(0.0,0.92), Q2=(0.0,−0.52)
Value
Cor
R2
Q2
c
patient.vs.control
−log10(pvalue)
log2(fold change)
alpha
0.4
DE_Meta
UP
DW
NoDiff
VIP
1
2
3
4
5
d
patient.vs.control_neg
Polycyclic aromatic hydrocarbon degradation
Naphthalene degradation
Drug metabolism - cytochrome P450
alpha-Linolenic acid metabolism
Benzoate degradation
Two-component system
Fluorobenzoate degradation
Dioxin degradation
Cholesterol metabolism
Aminobenzoate degradation
2-Oxocarboxylic acid metabolism
Chlorocyclohexane and chlorobenzene degradation
Biosynthesis of alkaloids derived from histidine and purine
Primary bile acid biosynthesis
Degradation of aromatic compounds
Citrate cycle (TCA cycle)
Carbon fixation pathways in prokaryotes
Caffeine metabolism
Bile secretion
Microbial metabolism in diverse environments
Terms
Ratio
-log10(Pvalue)
Number



Highlights

1.There is a significant difference in the gut microbial community composition and serum metabolites between POAG patients and healthy subjects.

2.Gut microbiota and its effects on host metabolism might be linked with the pathogenesis of glaucoma.

3.Gut microbiota and metabolites-targeted interventions of glaucoma should be considered in future.