ORIGINAL RESEARCH

# Untargeted Metabolomic Study of Patients with Wet Age-Related Macular Degeneration in Aqueous Humor

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**Purpose:** The objective of this study was to ascertain metabolic biomarkers and investigate the metabolic alterations associated with aqueous humor (AH) in wet age-related macular degeneration (AMD).

**Methods:** AH samples were collected from a total of 20 participants, including 10 individuals diagnosed with wet AMD and 10 individuals undergoing cataract surgery, serving as the control group. Metabolomics analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify and quantify metabolites.

**Results:** A total of 155 metabolites were identified in the AH samples. Among them, 10 metabolites emerged as potential biomarkers capable of differentiating patients with wet AMD from the control group. In the AH of wet AMD patients, there was increased expression of Cardiolipin (CL) (72:5), Diglyceride (DG) (18:3\_18:2), DG (36:5e) and Triglyceride (TG) (24:7), while the expression of Ceramides (Cer) (d32:0), Cer (d34:0), Cer (d36:0), Monogalactosyldiacylglycerol (MGDG) (16:1\_18:3), Sphingosine (SPH) (d18:0) and TG (16:0\_10:4\_16:0) was down regulated.

**Conclusion:** Through metabolomics analysis of AH, this study successfully uncovered valuable metabolic biomarkers linked to wet AMD. These findings contribute to a more comprehensive understanding of the pathogenesis of wet AMD and offer potential avenues for the development of innovative treatment strategies for this condition.

Keywords: metabolomic, wet age-related macular degeneration, aqueous humor

#### Introduction

Age-related macular degeneration (AMD) is a common cause of irreversible vision loss in individuals over the age of 50 and constitutes a significant concern in global public health.<sup>1,2</sup> AMD can be clinically classified into two forms: dry AMD and wet AMD. Wet AMD, accounting for 90% of blindness cases caused by AMD, is characterized by choroidal neovascularization (CNV).<sup>3,4</sup> Although the diagnosis of wet AMD is conclusive, there is still controversy surrounding the etiology and pathophysiological mechanisms of the condition.

Metabolomics is a research field involving the identification and quantification of small molecules (metabolites) in biological samples and has been utilized to study metabolic alterations associated with various diseases, offering insights into their underlying mechanisms.<sup>5,6</sup> Metabolomic analysis of aqueous humor (AH) has exhibited potential in revealing metabolic changes related to eye diseases.<sup>7,8</sup> Studies have indicated that myopic patients have significantly different metabolic profiles in AH compared to healthy controls.<sup>9</sup> Specifically, increased concentrations of 27 important metabolites were found in myopic patients.<sup>10</sup> These metabolites may provide valuable information about metabolic changes that occur in the eye during the development and progression of myopia. In glaucoma—a complex neurodegenerative disease affecting the optic nerve-metabolomic analysis of AH has revealed dysregulation of metabolites related to osmoprotection, neuroprotection, and amino acid metabolism.<sup>11</sup> These findings suggest that metabolomics might play a role in understanding the pathophysiology of glaucoma and in identifying biomarkers for

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disease progression. Additionally, in diabetic retinopathy (DR), a common complication of diabetes affecting the retina, metabolomics research on AH has identified differential metabolites.<sup>12</sup> These metabolites might be associated with the metabolic dysregulation and vascular dysfunction observed in DR. However, the application of metabolomics in investigating wet AMD remains limited, and further research is needed to elucidate specific metabolic changes associated with this condition.

Therefore, the goal of this study is to perform a comparative analysis between wet AMD patients and a control group to identify unique metabolic alterations. Furthermore, the study aims to identify potential biomarkers among these differentially regulated metabolites, which could help to better understand the pathophysiology of wet AMD and aid in the development of diagnostic and therapeutic strategies.

## **Materials and Methods**

#### Study Participants and Sample Collection

The study enrolled 20 subjects from December 1, 2021, to March 1, 2023. All study protocols adhered to the ethical principles outlined in the Declaration of Helsinki. The institutional Research Ethics Committee of the Tong Ren Hospital affiliated with the Shanghai Jiao Tong University School of Medicine granted ethical approval for the research (Approval Number: 2021-078-01). Comprehensive written informed consent was procured from all participants prior to sample collection. The acquisition of AH entailed the use of a 30-gauge needle to perform an anterior chamber paracentesis, followed by the aspiration of approximately  $50-100\mu$ L of the fluid. The sample was then deposited into an Eppendorf tube and preserved at  $-80^{\circ}$ C until further analysis. For patients with wet AMD, AH samples were collected before intravitreal injection of anti-vascular endothelial growth factor (anti-VEGF) drugs after surface anesthesia with proparacane hydrochloride ocular solution. The cataract patient received AH samples after surface anesthesia with proparacane hydrochloride ocular solution at the beginning of the surgery.

The study included a cohort of 10 patients (10 eyes) diagnosed with wet AMD, all of whom were receiving their first vitreous injection of anti-VEGF therapy. The diagnosis of wet AMD in individuals was established based on spectral-domain optical coherence tomography (SD-OCT) and indocyanine green angiography (ICGA) assessments. A control group was constituted of 10 age- and sex-matched individuals (10 eyes) who underwent cataract extraction but did not have wet AMD. Assessment of their cataracts was performed utilizing the Lens Opacities Classification System III (LOCS III), with each cataract in the control arm achieving a classification of N2C2P2. Diagnosis of nAMD was confirmed via spectral-domain optical coherence tomography (SD-OCT) coupled with indocyanine green angiography (ICGA). Exclusion criteria encompassed any other vitreoretinal pathology, active uveitis or ocular infection, primary or secondary glaucoma, corneal pathology, high myopia, prior retinal surgery, any anterior segment surgeries or intraocular laser procedures.

## Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) Analysis

The LC-MS/MS analytical procedure was conducted by Biotree Biotech Co., Ltd. (Shanghai, China) utilizing an ultrahigh-performance liquid chromatography (UPLC) platform equipped with a diode array detector (Thermo Fisher Scientific). Chromatographic separation was effected through a UPLC BEH Amide column  $(2.1\times100$ mm,  $1.7\mu$ m) interfaced with a Q Exactive HFX mass spectrometer (Thermo Fisher). The mobile phase was composed of a mixture containing 25 mmol/L ammonium acetate, 25 mmol/L ammonia solution at pH 9.75, and acetonitrile. The autosampler temperature was maintained at 4°C, with a set injection volume of 3  $\mu$ L. Selection of the Q Exactive HFX mass spectrometer was predicated on its capability to acquire tandem mass spectrometry (MS/MS) data in a data-dependent acquisition mode, which is governed by the Xcalibur software suite. This scheme enables successive full-scan MS spectrum analysis. The electrospray ionization (ESI) source parameters for the Q Exactive HFX were set as follows: sheath gas flow rate at 30 arbitrary units, auxiliary gas flow rate at 25 arbitrary units, capillary temperature at 350°C, mass resolution for full MS scan at 60,000, and a mass resolution for MS/MS scans at 7500, with collision-induced dissociation settings appropriately adjusted.

#### Data Processing

The raw MS data underwent conversion to MzXML files via ProteoWizard. Following this, XCMS was employed for feature detection, retention time correction, and alignment. Metabolite identification was facilitated by a combination of accurate mass (< 25 ppm) measurements and experimental MS/MS matching against our proprietary tandem MS spectral library, as well as other publicly available databases such as NIST and MassBank. During the extracted-ion feature analysis, metabolic peaks detected in less than 50% of all quality control (QC) samples were excluded. Only variable ion peaks exhibiting more than 50% of nonzero measurement values in at least one group were selected for subsequent statistical analysis.

For all multivariate data analyses and modeling, SIMCAP software (Version 14.0, Umetrics, Umeå, Sweden) was utilized. Data were mean-centered using Pareto scaling. Principal Component Analysis (PCA) served as an unsupervised learning approach to discern the overarching separation trends amongst the dataset. Subsequently, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was harnessed as a supervised learning framework to pinpoint metabolites that exhibited significant alterations across different sample cohorts. The descriptive performance of the models was assessed using cumulative  $R^2X$  and  $R^2Y$  values, with a perfect model achieving  $R^2X$  (cum) = 1 and  $R^2Y$  (cum) = 1. Prediction performance was evaluated using cumulative Q2 values and a permutation test (n=200). The permuted model should demonstrate an inability to predict classes, with R2 and Q2 values at the Y-axis intercept being lower than those of the non-permuted model. OPLS-DA facilitated the identification of discriminating metabolites based on variable importance on projection (VIP).

The P-value was determined using one-way analysis of variance (ANOVA) for multiple group comparisons. Metabolites with VIP values greater than 1.0 and P-values less than 0.05 were considered statistically significant. Fold change was computed as the logarithm of the average mass response (area) ratio between two arbitrary classes.

#### Statistical Analysis

Statistical analysis was conducted using SPSS (Version 19.0, Chicago, IL, USA). An independent *t*-test was employed to compare the means of continuous variables between groups, while categorical variables were compared using the chi-squared test. All data were assessed for Gaussian distribution. A threshold of P < 0.05 was adopted to denote statistical significance in the differential expression of metabolites.

## Results

#### Characteristics of the Participants

To elucidate the metabolic signatures associated with AH in wet AMD, a cohort study was conducted with meticulous recruitment of 10 age and sex-matched healthy controls and 10 patients diagnosed with wet AMD for comprehensive untargeted metabolomics assessments. Demographic and clinical variables such as age, sex, hypertension, coronary artery disease, and diabetes mellitus were homogenized across groups to ensure no significant confounding disparities were present (Table 1).

	Wet AMD (n=10)	Control (n=10)	P value
Gender (male/female)	5/5	4/6	0.653
Age (years), median	69.80±1.56	70.10±1.87	0.683
Hypertension (yes/no)	5/5	4/6	0.653
Diabetes (yes/no)	1/9	1/9	1
Coronary heart disease (yes/no)	1/9	1/9	1
Hyperlipidemia (yes/no)	5/5	3/7	0.361

Table I Clinical Characteristics of the Patients

**Note**: The data are presented as the mean±SD.

**Abbreviation**: AMD, age-related macular degeneration.

### **AH Metabolic Profiles**

In this study, we utilized untargeted metabolomic profiling to characterize the metabolite composition of AH specimens. The results of the PCA showed that one sample from both the control group and the experimental group was separated individually, while the other samples were closely clustered together (Figure 1). Subsequent multivariate analysis using OPLS-DA was implemented to decipher the metabolic disparity between the cohorts. The OPLS-DA score scatter plot (Figure 2) demonstrated a distinct segregation between the wet AMD patients and the control subjects, indicating the potential metabolic perturbations associated with wet AMD. The validity and predictive accuracy of the OPLS-DA model were further substantiated by a permutation testing procedure, where both R2Y and Q2 values for permuted datasets were observed to be considerably lower than the corresponding values obtained from the actual data (Figure 3), corroborating the model's non-random predictive strength and affirming its statistical robustness.

#### Identification of Potential Biomarkers

Relative to the control group, our analysis delineated a set of 10 metabolites that exhibited significant differential abundance in the wet AMD group (VIP>1 and p<0.05) (Table 2). Analyze through volcano plots and different lipid categories (Figure 4), to effectively discern the magnitude of change and the significance of these altered metabolites. Figure 4A illustrates the differential lipid expression between the wet AMD and the control group. Figure 4B presents an overview of all lipid classes in both the wet AMD and control groups. Figure 4C highlights the differences in lipid classes between the wet AMD and control groups. This was complemented by the construction of a heat map coupled with hierarchical clustering analysis (Figure 5), which facilitated the differences in metabolite expression and different lipid categories between the two groups. Specifically, Figure 5A illustrates the differential lipid expression between the wet AMD group and the control group,



Figure 1 The score plot of PCA analysis.



Figure 2 The score plot of the PLSDA model.



Figure 3 Validation plots for the OPLS-DA mode.

Metabolites	RT (s)	VIP	Fc	p value	Up/Down Regulation	
CL (72:5)	5.339	1.96245889	1.24	0.0397827	Up	
DG (18:3_18:2)	13.0632501	1.83789097	1.47	0.03671985	Up	
DG (36:5e)	26.4770577	1.94697868	1.35	0.03894942	Up	
TG (24:7)	1.32951553	1.97555713	1.29	0.04710967	Up	
Cer (d32:0)	10.7876113	2.31455417	0.67	0.01166562	Down	
Cer (d34:0)	12.4668284	2.3348134	0.72	0.01133351	Down	
Cer (d36:0)	14.1201165	1.77467762	0.77	0.04834021	Down	
MGDG (16:1_18:3)	11.4565099	2.82701178	0.07	0.00060499	Down	
SPH (d18:0)	1.965	2.65163683	0.68	0.0027554	Down	
TG (16:0_10:4_16:0)	5.30145632	2.27384064	0.56	0.0114779	Down	

 $\label{eq:controls} \textbf{Table 2} \ \text{List of Significantly Different Metabolites in AH from Wet AMD Compared with Controls}$ 

Abbreviations: CL, Cardiolipin; DG, Diglyceride; TG, Triglyceride; Cer, Ceramides; MGDG, Monogalactosyldiacylglycerol; SPH, Sphingosine.

while Figure 5B highlights the differences in lipid class distributions between these groups. Through these analytical approaches, it became evident that wet AMD was characterized by an up-regulation of 4 metabolites and a concurrent down-regulation of 6 metabolites, when juxtaposed with the control group, indicating a markedly altered metabolic milieu in wet AMD patients.



Figure 4 Volcano plots of aqueous humor metabolomic. (A) Differential lipid expression between wet AMD and control group, (B) All lipid classes in wet AMD and control group, (C) Differential lipid classes between wet AMD and control group.



Figure 5 Heat plot of aqueous humor metabolomic. (A) Differential lipid expression between wet AMD and control group, (B) Differential lipid classes between wet AMD and control group.

#### Discussion

In this study, untargeted metabolomics analysis was employed to characterize the metabolite composition of AH samples from patients with age-related wet AMD. PCA results showed that one sample from each of the control and experimental groups was individually separated, while the remaining samples were closely clustered together (Figure 1). Subsequent multivariate analysis using OPLS-DA was conducted to reveal metabolic differences between the two cohorts. The OPLS-DA score scatter plot (Figure 2) demonstrated a distinct segregation between wet AMD patients and control subjects, indicating potential metabolic perturbations associated with wet AMD. The validity and predictive accuracy of the OPLS-DA model were further confirmed through permutation testing. Both R2Y and Q2 values for permuted datasets were significantly lower than those obtained from the actual data (Figure 3), confirming the non-random predictive capability and statistical robustness of the model.

In a previous study, it was found that 18 metabolites were significantly altered in patients with wet AMD. These included changes in amino acid metabolism associated with mitochondrial dysfunction (L-proline), energetic substrates (carnitine, deoxycarnitine, TML), and compromised carbohydrate metabolism (cis-aconitic acid and itaconate acid).<sup>13</sup> Previous global metabolomics research has indicated higher levels of L-proline in early AMD and lower levels in intermediate or late AMD, as well as a significant reduction in L-proline concentration in the AH of wet AMD.<sup>14</sup> Additionally, a study have suggested that proline is the preferred nutrient substrate in human retinal pigment epithelial (RPE) cells, and exporting proline-derived mitochondrial products for use in the peripheral retina helps to combat oxidative damage.<sup>15</sup> However, our analysis revealed a set of 10 metabolites that showed significant differential abundance in the wet AMD group compared to the control group (VIP>1 and p<0.05) (Table 2). We have detected high expression of Cardiolipin (CL) (72:5), Diglyceride (DG) (18:3–18:2), DG (36:5e) and Triglyceride (TG) (24:7) in the AH of wet AMD patients. CL is a crucial lipid molecule that plays a key role in the structure and function of cell membranes, particularly in the retina.<sup>16</sup> Accumulation of phosphatidylcholine in the retina may trigger inflammatory responses and oxidative stress.<sup>17</sup> In our study, we also found elevated expression of CL in wet AMD, which may be a key factor in the development of AMD. DG is lipid molecules found in the bloodstream, typically associated with conditions such as obesity and cardiovascular disease. However, their specific role in AMD is not yet clear. Some studies suggest that elevated levels of DG in the blood may be associated with an increased risk of AMD, possibly due to their role in promoting inflammation and oxidative stress, known factors in the development and progression of AMD.<sup>18,19</sup> Our research found increased expression of DG in patients with wet AMD, suggesting that regulating DG levels could be a potential therapeutic strategy for AMD. Ceramides (Cer) are a type of sphingolipid that have been implicated in various neurodegenerative diseases. Research suggests that gangliosides, another type of sphingolipid, are important for the structure and function of the retina, but circulating gangliosides appear to be unrelated to retinal damage occurring in the process of AMD.<sup>20</sup> Our study found that Cer (d32:0), Cer (d34:0) and Cer (d36:0) were down regulated in the AH of patients with wet AMD, suggesting a potential association between Cer and the development of AMD. Currently, there is scarce research and discussion on the correlation between Monogalactosyldiacylglycerol (MGDG) and AMD, or Sphingosine (SPH) and AMD. Our study findings suggest a reduction in MGDG (16:1 18:3) and SPH (d18:0) levels in cases of wet AMD. TG is a type of fat molecule that typically exists in the blood. Some studies have indicated a correlation between elevated triglyceride levels and retinal artery sclerosis and retinal arteriolar occlusion,<sup>21,22</sup> which may be a risk factor for wet AMD. Our research findings indicate an elevation in TG (24:7) levels in the AH of wet AMD, while TG (16:0 10:4 16:0) levels are decreased in the AH of wet AMD. Further discussion of these findings suggests that these significantly altered metabolites could be potential biomarker candidates, aiding in a better understanding of the pathological processes and related metabolic disruptions in wet AMD. For example, up-regulated metabolites may be associated with pathological processes such as inflammation and oxidative stress, while downregulated metabolites may involve critical biological functions related to energy metabolism and cell apoptosis. These results provide valuable clues for further exploration of the pathogenesis of wet AMD and the identification of therapeutic targets. Volcano plots and analysis of different lipid categories effectively distinguished the magnitude of change and significance of these altered metabolites (Figure 4). This conclusion was supported by constructing a heat map coupled with hierarchical clustering analysis (Figure 5), which highlighted the differences in metabolite expression and the differential lipid categories between the two groups.

However, there are some limitations to this study. The sample size was relatively small, and only specific metabolomics analysis was performed. Further research is warranted to expand the sample size and incorporate other analytical techniques such as genomics and proteomics to comprehensively and systematically elucidate the metabolic features associated with wet AMD. Additionally, the establishment of animal models and experimental validation are important directions for future studies to verify the reliability and corresponding functions of these potential biomarkers.

## Conclusion

Our study indicate a complex and severe metabolic disruption occurring in the AH of these patients. Additionally, we observed uncovered valuable metabolic biomarkers linked to wet AMD. These findings may shed light on potential prognostic metabolic biomarkers and novel therapeutic strategies for the prevention or delay of wet AMD development.

## Ethics Approval and Consent to Participate

All procedures of this study were in accordance with the tenets of the Declaration of Helsinki and approved by the medical ethics committee. Samples were collected after patients' written informed consent to participate in the study.

## **Patient Consent for Publication**

All Patients declare that they consent for publication.

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## Disclosure

The authors declare that they have no competing interests.

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