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Metabolic characteristics of Qi-Yin deficiency and heat stagnation in liver meridian patterns of dry eye based on tear metabolomics

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ABSTRACT

Objective To explore the metabolic differences between dry eye patients with Qi-Yin deficiency and heat stagnation in liver meridian patterns, and clarify their metabolic characteristics.

Methods Patients with dry eye who were treated in the Ophthalmology Ward and Outpatient Department of the First Hospital of Hunan University of Chinese Medicine from October 1, 2020, to October 30, 2021 were enrolled as the research participants in the study. They were assigned to two groups based on traditional Chinese medicine (TCM) syndrome types: heat stagnation in liver meridian pattern group and Qi-Yin deficiency pattern group. Healthy volunteers who underwent health check-ups in the Health Management Department were included as healthy group following the random number table method. The tears of the patients and the healthy volunteer participants were tested by high-performance liquid chromatography-mass spectrometry (LC-MS). The differential metabolites were screened out by multivariate statistical analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was performed on the differential metabolites. Finally, the association analysis of differential proteins and metabolites was conducted to verify and supplement the metabolites.

Results A total of 32 dry eye patients were enrolled, including 16 cases with heat stagnation in liver meridian pattern and 16 cases with Qi-Yin deficiency pattern. Fourteen healthy volunteers were included as healthy group. There were no significant differences in baseline characteristics among the three groups ($P > 0.05$). A total of 412 biomarkers were determined in Qi-Yin deficiency pattern group, mainly including lipids, lipid-like molecules, organic acids and their derivatives, organic heterocyclic compounds, and nucleosides and their analogues. For heat stagnation in liver meridian pattern group, 112 metabolites were determined, mainly including organic acids and their derivatives, lipids, and lipid-like molecules. The KEGG enrichment results of pathways and the relative content analysis of differential markers demonstrate that purine metabolism and caffeine metabolism pathways are common metabolic characteristics of all dry eyes. Among them, deoxyinosine monophosphate (dIMP) and 2-(formamido)-N1-(5-phospha-D-ribosyl) acetamidine can serve as their biomarkers. The main characteristics of Qi-Yin deficiency syndrome pattern were the significant enhancement of

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metabolic pathways such as lysine degradation, ovarian steroidogenesis, cholesterol metabolism, pyrimidine metabolism, and bile secretion ($P < 0.05$). Dry eye associated with the heat stagnation in liver meridian pattern is mainly characterized by inhibition of the valine, leucine, and isoleucine biosynthesis pathways ($P < 0.05$).

Conclusion Metabolomics can be used as an effective basis for TCM syndrome classification. Different patterns of dry eye syndrome exhibit typical characteristics in the types and concentrations of metabolites, which correspond to the syndrome classification in TCM. This study initially confirms the rationality of TCM syndrome classification and provides significant reference for the mechanism of dry eye and drug development.

1 Introduction

Dry eye is a multifactorial disease characterized by decreased stability of the tear film and accompanied by ocular discomfort, with complex etiology and pathophysiological mechanisms [1]. The incidence of dry eye in China has reached 21% to 30%, and the demand for its treatment is also increasing year by year [2]. Dry eye is associated with various systemic and metabolic conditions and functional disorders, including vitamin A deficiency, inadequate omega-3 and omega-6 fatty acid intake, ovarian dysfunction, menopause, diabetes, sarcoidosis, and the use of systemic drugs such as antidepressants, antihistamines, beta-blockers, and antidiuretics [3]. Although dry eye is closely related to many diseases, little is known about its molecular biological basis.

Traditional Chinese medicine (TCM) classifies dry eye into categories such as “white xerotic syndrome” “dryness and blurred vision syndrome” and “impending desiccation of spirit water”. Meanwhile, modern TCM diagnostic methods divide dry eye into various syndromes, including insufficiency of lung Yin, heat stagnation in liver meridian, lingering evil heat, and Qi-Yin deficiency [4]. Among these, the excess syndrome is mainly characterized by heat stagnation in liver meridian, while the deficiency syndrome is mainly characterized by the Qi-Yin deficiency, demonstrating an increasing trend [5]. Different syndromes require corresponding drug treatments, and their prescriptions are completely varied. Understanding the molecular biological basis of different syndromes of dry eye using modern methods is of great importance for developing diagnostic criteria and detection methods for various syndromes of dry eye.

Advanced high-throughput omics methods (genomics, transcriptomics, proteomics, and metabolomics) have developed greatly, which strengthened our understanding of complex diseases markedly. For example, VEHOF et al. [6] used gas chromatography and liquid chromatography coupled with mass spectrometry to analyze serum samples from 2 819 individuals. Among the 222 metabolites detected, a significant correlation was found between all five androgens and dry eye, leading authors to suggest 1-palmitoyl-sn-glycero-3-phosphocholine as a potential biomarker. Furthermore, the use of nano-liquid chromatography and quadrupole time-of-flight tandem mass spectrometry (nano-LC-Q-TOF-MS/MS) and ultra-performance LC-Q-TOF-MS/MS (UPLC-Q-TOF-MS/MS) techniques in proteomics and metabolomics analyses of tear fluid from patients with dry eye identified 34 metabolites as markers. These were notably related to proteins involved in immune and inflammatory processes. Concurrently, pathways including the complement and coagulation cascade, glycolysis/gluconeogenesis, and amino acid metabolism were associated with dry eye [7]. These findings not only provide diagnostic and therapeutic references for potential biomarkers of dry eye but also promote our understanding of its physiological and pathological mechanisms. However, there is a significant gap in research exploring the molecular biological underpinnings of dry eye across various TCM syndromes.

Therefore, this study aimed to investigate the metabolic profiles in tears of dry eye patients with either heat stagnation in liver meridian pattern or Qi-Yin deficiency pattern using non-targeted metabolomics (UPLC-Q-TOF-MS/MS) and protein-metabolite integrated analysis.

2 Materials and methods

2.1 General information

Patients with dry eye who were treated in the Ophthalmology Ward and Outpatient Department of the First Hospital of Hunan University of Chinese Medicine from October 1, 2020, to October 30, 2021 were enrolled as the research participants in the study. They were assigned to two groups based on TCM syndrome types: heat stagnation in liver meridian pattern group and the Qi-Yin deficiency pattern group. Healthy volunteers who underwent health check-ups in the Health Management Department were included as healthy group using a random number table method. All patients voluntarily participated in the study and signed the written informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the First Hospital of Hunan University of Chinese Medicine (HN-LL-SWST-2020-10), and

registered with the Chinese Clinical Trial Registry (ChiCTR2100046380). Data sharing is available for this study.

2.2 Diagnostic criteria

2.2.1 Western medicine diagnostic criteria for dry eye In line with the Chinese Consensus on the Treatment of Dry Eye (2020) [2], the diagnosis of dry eye is based on the following criteria: (i) the main complaint includes one or more symptoms such as eye fatigue, blurred vision, foreign body sensation, or pain; (ii) tear film break-up time (BUT) is ⩽ 10 s or Schirmer I test (SIT) ⩽ 2 mm/min; (iii) corneal staining assessed with sodium fluorescein shows more than 5 staining points. A diagnosis can be established if any one of these criteria is met.

2.2.2 TCM syndrome differentiation criteria Based on *Traditional Chinese Medicine and Ophthalmology* [8], the diagnostic criteria of dry eye are as follows.

(i) Heat stagnation in liver meridian pattern of dry eye. (a) Main symptoms: dryness and burning pain in the eyeballs, slight redness of the white part of the eye, nebulae on the black part of the eye, or intolerance to prolonged viewing. (b) Secondary symptoms: bitter taste in the mouth, dry throat, irritability/easy anger, insomnia/frequent dreams, dry stool, yellow urine; tongue red with thin yellow or thick yellow fur, thready or deep and thready pulse.

(ii) Qi-Yin deficiency pattern of dry eye. (a) Main symptoms: dryness and discomfort in the eyes, lack of luster in the eyes, frequent blinking, photophobia, slight redness of the white part of the eye, intolerance to prolonged viewing, worsening symptoms after prolonged viewing, potential blurring of vision, potential nebulae or filaments on the black part of the eye, difficulty in healing. (b) Secondary symptoms: dry mouth with little saliva, fatigue, dizziness, tinnitus, weakness in the lower back and knees; tongue light red with thin fur, pulse thin or deep and thin.

2.3 Inclusion and exclusion criteria

2.3.1 Inclusion criteria for patients with dry eye (i) People who met the above diagnostic criteria; (ii) age between 18 and 75 years; (iii) signed informed consent.

2.3.2 Exclusion criteria for patients with dry eye (i) Patients with other ocular diseases, such as blepharitis and congenital anhidrosis; (ii) patients with a history of primary liver, kidney, heart, or lung diseases; (iii) patients with immune system abnormalities, such as Sjögren's syndrome and systemic lupus erythematosus; (iv) patients who have undergone eye surgery in the past three months; (v) patients who have used drugs affecting tear secretion within the past one month.

2.3.3 Inclusion criteria for healthy group (i) Healthy individuals without ocular surface symptoms, visual function symptoms, or ciliary muscle irritation symptoms; (ii) normal anterior segment tissue on slit lamp examination; (iii) BUT > 10 s; (iv) SIT > 2 mm/min; (v) signed informed consent.

2.3.4 Exclusion criteria for healthy group (i) Symptoms such as dryness, foreign body sensation, fatigue, itching, and swelling in the ocular surface; (ii) symptoms of ciliary muscle irritation like photophobia, pain, tearing, redness of the eye, and stinging sensation; (iii) symptoms of visual function abnormalities such as blurred vision; (iv) a history of meibomian gland dysfunction and dry eye; (v) meeting any of the exclusion criteria for dry eye.

2.4 Instruments and reagents

Methanol, formic acid, and acetonitrile were purchased from Thermo Fisher Scientific, USA. L-2-chlorophenylalanine was purchased from Shanghai Hengchuang Biotechnology Co., Ltd., China. All chemicals and solvents were of analytical or chromatographic grade. Instruments were purchased from the following manufacturers: ultrasonic cleaner (F-060SD, Shenzhen Fuyang Technology Group Co., Ltd.); vortex oscillator (TYXH-I, Shanghai Hanuo Instrument Co., Ltd.); desktop high-speed freezing centrifuge (TGL-16MS, Shanghai Luxiangyi Centrifuge Instrument Co., Ltd.); high-resolution mass spectrometer (QE, Thermo Fisher Scientific); high-performance liquid chromatograph (ACQUITY UPLC I-Class PLUS, Waters); chromatographic column (ACQUITY UPLC HSS T3,100 mm × 2.1 mm, 1.8 μm; Waters).

2.5 Tear collection

Before collecting specimens, patients were asked to rest in the examination restroom for 15 min and avoid rubbing their eyes. The capillary glass tube method was employed, with a precise quantitative glass capillary gently contacting the lower eyelid margin tear triangle area [9]. The tear fluid entered the tube through capillary action, and then the collected tear fluid was transferred to a 0.2 mL Eppendorf (EP) tube. The amount of tear fluid collected was approximately 50 μL, and the tear fluid samples were stored in a – 80 °C freezer.

2.6 Metabolomics method study

2.6.1 Pretreatment The samples designated for testing, previously stored at – 80 °C were thawed at 4 °C. Each sample was transferred 20 μL, and 10 μL of internal standard (L-2-chlorophenylalanine, 0.06 mg/mL; methanol as solvent) was added, vortexed (2 600 rpm, 4 °C, 10 s); 150 μL of methanol-water (V : V = 4 : 1) was added, vortexed for 1 min; ultrasonic extraction in an ice water bath for 10 min, left at – 20 °C for 30 min. After centrifugation

(13 000 rpm at 4 °C for 10 min), the supernatant was taken for liquid chromatography-mass spectrometry (LC-MS) analysis. Quality control samples were prepared by mixing equal volumes of extracts from all samples.

2.6.2 LC-MS analysis conditions The mobile phase consisted of A-water (containing 0.1% formic acid) and B-acetonitrile (containing 0.1% formic acid). The initial gradient of the chromatographic conditions was set at 95% A, held for 2 min, then decreased to 70% A at 4 min, to 50% A at 8 min, to 20% A at 10 min, and to 0% A at 14 min, where it was held for an additional 1 min. The column temperature was maintained at 45 °C, with a flow rate of 0.35 mL/min and an injection volume of 5 μL. The mass spectrometer ion source was electrospray lonization (ESI), set at a temperature of 200 °C, and the sample mass spectrometry signal acquisition was performed in both positive (ESI+) and negative (ESI–) ion scanning modes. The mass spectrometry acquisition range was set from 100 to 1 200 m/z, with a full scan resolution of 70 000 and a secondary mass spectrometry resolution of 17 500. The spray voltage for positive ions was 3 800 V, and for negative ions, it was – 3 200 V. The sheath gas flow rate was 40 Arb, the auxiliary gas flow rate was 10 Arb, and the ion transfer tube temperature was 320 °C.

2.6.3 Metabolomics workflow In this study, tear samples were collected from participants and analyzed using the ultra performance liquid chromatography-quadrupole/electrostatic field orbitrap mass spectrometry (UHPLC-QE MS) platform for ESI+ and ESI– modes to obtain data. The raw data were processed using the metabolomics processing software Progenesis QI V2.3 for baseline filtering, peak identification, integration, retention time correction, peak alignment, and normalization. The identification of compounds is based on accurate mass numbers, secondary fragments, and isotopic distributions. Qualitative analysis is performed using databases such as the Human Metabolome Database (HMDB, http://www.hmdb.ca/), LipidMaps, METLIN, and in-house libraries. Compounds are then scored based on the qualitative results, with a screening criterion of 36 points (out of a maximum of 60 points). Compounds with scores below 36 are considered inaccurately identified and are removed. For the extracted data, ion peaks with intra-group missing values (0-values) > 50% are deleted, and 0-values are replaced with half of the minimum value. Finally, the positive and negative ion data are combined into a single data matrix table, which contains all the information extracted from the raw data that can be used for analysis. Multivariate statistical analysis first employs unsupervised principal component analysis (PCA) to observe the overall distribution between samples and the stability of the entire analysis process, followed by supervised orthogonal partial least squares discriminant analysis (OPLS-DA) to distinguish the overall differences in metabolic profiles between groups, identifying differentially expressed metabolites. A combination of multi-dimensional and single-dimensional analysis methods is used to screen for differentially expressed metabolites between groups. Metabolites are extracted based on fold change (FC) > 1.2 and $P < 0.05$ criteria, and visualized using volcano plots and heat maps. Metabolites with significant differences are then selected using a variable importance in projection (VIP) threshold > 1 and $P < 0.05$, and pathway analysis is performed. This study utilizes the MetaboAnalyst 5.0 online data analysis website (https://www.metaboanalyst.ca/) for analysis, which is a tool for pathway enrichment exploration and visualization of changes in metabolites. Selected marker metabolites undergo KEGG pathway enrichment analysis, and pathways are selected based on enrichment factors and *P*-values, with a criterion of $P < 0.05$. The research flow chart is shown in Figure 1.

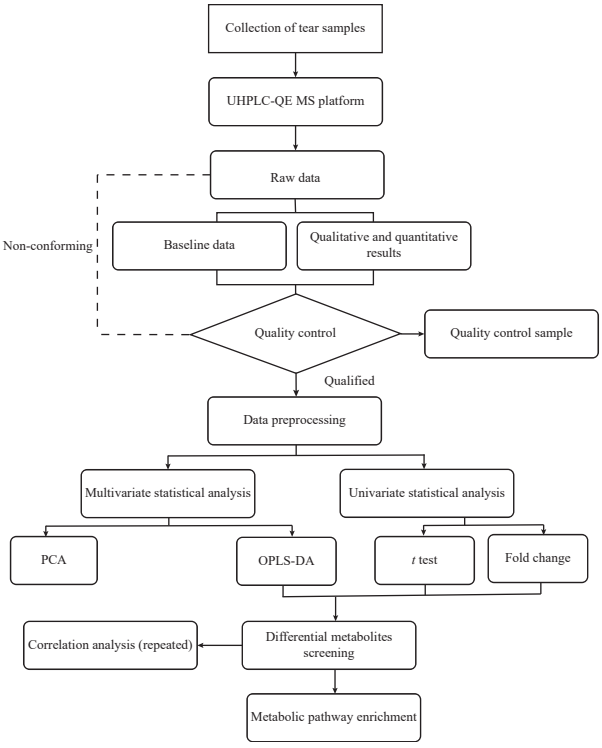

Figure 1 Workflow diagram of metabolomics

2.7 Statistical analysis

Statistical analysis was performed using SPSS 26.0. Measurement data that followed a normal distribution were presented as mean ± standard deviation (SD). The *t* test was used for testing methods with a significance level of $\alpha = 0.05$. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Baseline data

In this experiment, a total of 32 dry eye patients were enrolled, including 16 cases with heat stagnation in liver

meridian pattern group and 16 cases with Qi-Yin deficiency pattern group. Fourteen healthy volunteers were included as healthy group. No statistically significant differences were observed in baseline data (age, gender, lifestyle, medication method, and disease course) among the three groups of participants ($P > 0.05$).

3.2 Multivariate statistical analysis

The metabolic data of the three groups were obtained via the LC-MS analysis, covering all metabolites under both ESI+ ion mode and ESI– ion mode. A total of 12 238 positive ion characteristic peaks (3 556 metabolites) and 5 834 negative ion characteristic peaks (1 122 metabolites) were identified, mainly including lipids, lipid molecules, organic acids, and their derivatives (Figure 2A and 2B). A principal component analysis showed that the Qi-Yin deficiency and the heat stagnation in liver meridian patterns of dry eye were clearly separated from healthy group in the metabolic profile, located in the top left and bottom right corners, respectively (Figure 2C). The OPLS-DA model further confirmed this difference, with the *R2Y* value of 0.935 and *Q2* value of 0.644 for the Qi-Yin deficiency type of dry eye versus the control group (Figure 2D), and the *R2Y* value of 0.96 and *Q2* value of 0.47 for the heat stagnation in liver meridian type of dry eye versus the control group (Figure 2E), indicating the reliability of the two established OPLS-DA models. Furthermore, the OPLS-DA model between the Qi-Yin deficiency and the heat stagnation in liver meridian patterns of dry eye patients also met the requirements and could be used for further screening of differential metabolites and analysis of metabolic characteristics (Figure 2F).

3.3 Identification of differential metabolites

This study extracted metabolites with significant differences and visualized them using volcano plots (Figure 3A and 3B). Overall, 412 markers were identified and confirmed in the comparison between the Qi-Yin deficiency pattern and healthy group, including 294 and 118 metabolites from ESI+ and ESI– models, respectively (Figure 3C). According to the chemical classification method in the HMDB, they were divided into 10 subcategories, among which the significantly different metabolites mostly included lipids, lipid-like molecules, organic acids and their derivatives, organic heterocyclic compounds, nucleosides, nucleotides, and analogs.

Similarly, the markers, based on the above criteria, obtained from the comparison model between the heat stagnation in liver meridian pattern and healthy group were identified and confirmed. The comparison between the heat stagnation in liver meridian pattern and healthy group confirmed 112 metabolites, including 89 and 23 metabolites from ESI+ and ESI– models, respectively

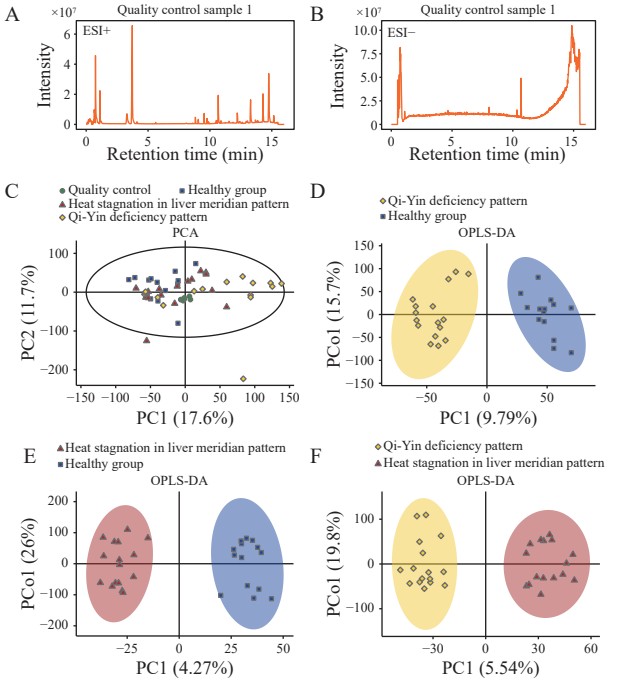

Figure 2 Representative LC-MS spectrum and multivariate statistical analysis for tear samples

A and B, LC-MS analysis plot of ESI+ and ESI–, respectively. C, PCA score plot. D, OPLS-DA model between the Qi-Yin deficiency pattern and healthy groups. E, OPLS-DA model between heat stagnation in liver meridian pattern and healthy groups. F, OPLS-DA model between Qi-Yin deficiency pattern and heat stagnation in liver meridian pattern groups.

(Figure 3D). They were divided into 11 subcategories following the chemical classification method in the HMDB, mainly including organic acids and their derivatives, lipids, and lipid-like molecules.

To further demonstrate the common metabolic characteristics of patients with Qi-Yin deficiency and heat stagnation in liver meridian patterns of dry eye, we extracted and analyzed the common differential metabolites between these two syndrome patterns of dry eye and healthy group. The results showed 32 common differential metabolites in both syndrome patterns of dry eye patients, which were mainly concentrated in the categories of lipids, lipid-like molecules, organic acids, and derivatives of organic acids. Further analysis of these common differential metabolites indicated that their expression trends were consistent in both patterns of dry eye, suggesting that they may play an important role in its pathological mechanism.

3.4 Metabolite-related and KEGG pathway enrichment analyses

The interrelationships among significantly differential metabolites were further studied, and KEGG pathway enrichment analysis was performed on the selected marker metabolites. Compared with healthy group, the Qi-Yin deficiency pattern group has enriched seven metabolic

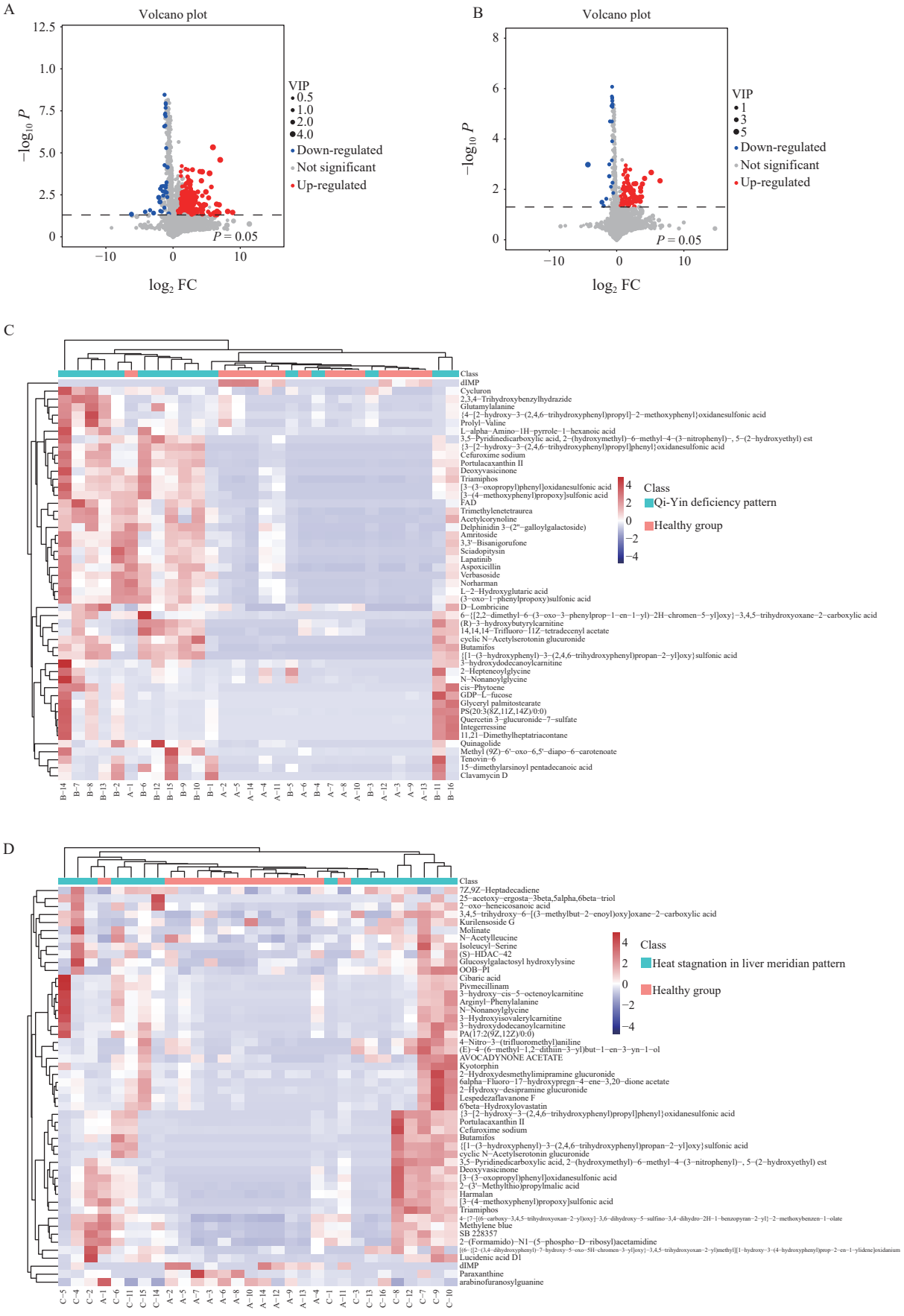

Figure 3 Volcano plots and heatmaps of differential metabolites in different dry eye syndromes

A and B, volcano plots for the Qi-Yin deficiency pattern and heat stagnation in liver meridian pattern groups compared with healthy group, respectively. C and D, differential metabolite hotspots (the top 50) for the Qi-Yin deficiency pattern and heat stagnation in liver meridian pattern groups compared with healthy group, respectively.

pathways: lysine degradation, ovarian steroidogenesis, cholesterol metabolism, pyrimidine metabolism, bile secretion, purine metabolism, and caffeine metabolism pathways (Figure 4A). Compared with healthy group, the heat stagnation in liver meridian pattern group has enriched three metabolic pathways: caffeine metabolism, purine metabolism, and valine, leucine, and isoleucine biosynthesis pathways (Figure 4B). The results suggest that purine metabolism and caffeine metabolism pathways may be common pathways in the tear fluid of dry eye; however, the Qi-Yin deficiency pattern and the heat stagnation in liver meridian pattern of dry eye have distinct metabolic characteristics. For example, the typical feature of the heat stagnation in liver meridian pattern of dry eye denotes the difference in valine, leucine, and isoleucine biosynthesis pathways, while the Qi-Yin deficiency pattern of dry eye has a greater variety of different metabolites in the tear fluid.

To further confirm the common metabolic characteristics of dry eye and the metabolic characteristics of the Qi-Yin deficiency and the heat stagnation in liver meridian patterns, this study plotted the relative content graphs of the main differential metabolites in the above-mentioned enriched metabolic pathways. The dIMP and 2-(formamido)-N1-(5-phospho-D-ribosyl) acetamidine, which belong to purine metabolism, showed a consistent trend of change in both patterns, with a significant decrease in deoxyinosine (dIMP) ($P < 0.05$) and a significant increase in 2-(formamido)-N1-(5-phospho-D-ribosyl) acetamidine ($P < 0.05$) in dry eye (Figure 4C). For the heat stagnation in liver meridian pattern of dry eye, the typical characteristics contained a significant decrease in caffeine and paraxanthine, which belongs to caffeine metabolism ($P < 0.05$, Figure 4D), indicating that the inhibition of the caffeine metabolism pathway is one of its typical characteristics. While, the increase in 2-amino-3-methylbutanoic acid, which is a part of the valine, leucine, and isoleucine biosynthesis pathway, indicated that the valine, leucine, and isoleucine biosynthesis pathway may be enhanced in the heat stagnation in liver meridian pattern of dry eye. In the Qi-Yin deficiency pattern of dry eye, a relatively consistent trend was found, except for purine metabolism, in five specific pathways: lysine degradation, ovarian steroidogenesis, cholesterol metabolism, pyrimidine metabolism, and bile secretion, where metabolites mainly showed an upward trend, indicating a potentially significant increase in these metabolic pathways in the Qi-Yin deficiency pattern of dry eye ($P < 0.05$).

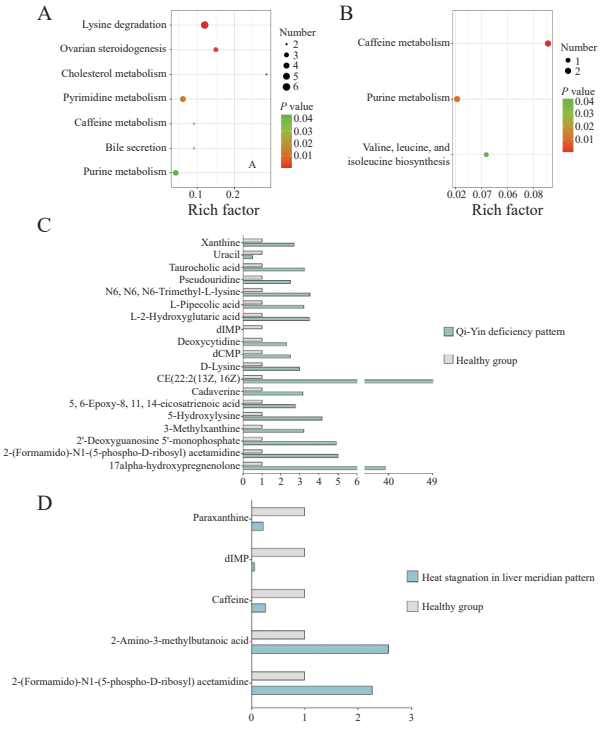

Figure 4 Metabolic pathway enrichment analysis of differential metabolites in different patterns of dry eye syndromes

A, metabolic pathway enrichment analysis for the Qi-Yin deficiency pattern. B, metabolic pathway enrichment analysis for the heat stagnation in liver meridian pattern. C, content of major differential metabolites in the Qi-Yin deficiency pattern. D, content of major differential metabolites in the heat stagnation in liver meridian pattern.

4 Discussion

4.1 Exploring the different patterns of dry eye syndrome from a metabolic perspective

TCM theory holds that dry eye is caused by both internal and external factors, such as emotional distress, improper diet, excessive fatigue, and prolonged visual strain, causing an imbalance of Yin and Yang, disharmony of Qi and blood, and dysfunction of the internal organs. These internal and external factors affect the normal production and metabolism of tears, thereby causing dry eye. Modern TCM, based on different causes and symptoms, divides it into different types of treatment. Among them, patients with heat stagnation in liver meridian pattern of dry eye may demonstrate changes in the quality of tears, such as increased viscosity and poor excretion. At this time, metabolites in tears may show an increase in inflammatory markers and cytokines, causing metabolic disorders [10]. Patients with a Qi-Yin deficiency pattern of dry eye may have insufficient tear secretion. Meanwhile, the antioxidant substances in tears decrease, causing the eyes more susceptible to damage from free radicals [11]. Dry eye is a multifactorial disease of tears and the ocular surface. The key to treating dry eye is to address the main pathogenic mechanisms and resolve secondary mechanisms [12]. By analyzing the metabolites in the tears of patients with different pattern, results showed that they can play a beneficial role in more accurately determining the

patient's type via TCM, thereby formulating personalized treatment plans. At the same time, it is conducive to deeply understanding the pathogenesis of dry eye, which is beneficial for the development of related drugs.

In this study, compared with patients with the heat stagnation in liver meridian pattern of dry eye, patients with the Qi-Yin deficiency pattern of dry eye showed more changes in tear metabolites, with seven related metabolic pathways: lysine degradation, ovarian steroidogenesis, cholesterol metabolism, pyrimidine metabolism, bile secretion, purine metabolism, and caffeine metabolism pathways. Abnormal lysine degradation and cholesterol metabolism disorders are closely correlated with decreased tear secretion. Lysine is an amino acid, and its metabolites may cause oxidative stress, damaging the lacrimal and meibomian glands, thereby causing insufficient tear secretion and then symptoms of dryness and lack of luster in the eyes [13]. Cholesterol, an important component of meibomian gland secretions, helps maintain the lubrication and health of the ocular surface. This study found that the disorder of cholesterol secretion and excretion indicates a poor lubrication state of the ocular surface, which is similar to the clinical manifestations of dry eye. Simultaneously, ovarian steroidogenesis requires cholesterol as a precursor substance, and metabolic disorders may affect the supply and conversion of cholesterol, affecting the generation of ovarian steroids consequently [14]. The interaction between the two can significantly affect the lubrication and health of the ocular surface.

In addition, bile acids, as part of tears, maintain the lubrication and protection of the ocular surface. Bile plays a role in fat metabolism, and fat is an important component of cell membrane structure and function [15]. Insufficient bile secretion in the Qi-Yin deficiency pattern may lead to malnutrition of ocular tissues, affecting the clarity of vision. Moreover, abnormalities in the purine and caffeine metabolism may result in an insufficient cellular energy supply, which have an impact on the function of ocular tissues. Abnormalities in the purine and caffeine metabolism involve cellular energy metabolism disorders, influencing the normal function of the lacrimal glands, causing discomfort in the eyes, photophobia, and other symptoms. Dry eye symptoms, especially after prolonged use of the eyes, may worsen, which is related to insufficient cellular energy supply and inflammatory reactions. Abnormalities in the pyrimidine, purine, and caffeine metabolism are related to the presence of mitochondrial dysfunction in dry eye patients [16]. Mitochondria serves as the main site of energy production within cells. Abnormalities in the metabolism of pyrimidine, purine, and caffeine can impair mitochondrial function, which in turn affects cellular energy supply. This impairment, including the energy needs of the lacrimal and meibomian glands, can exacerbate the occurrence and development of dry eye.

In summary, abnormalities in the metabolism of pyrimidine, purine, and caffeine reduce the energy supply of the human body, leading to Qi deficiency. Lysine degradation, ovarian steroidogenesis, cholesterol metabolism, and bile secretion affect the secretion of human Yin fluids, which demonstrates Yin deficiency. Therefore, the abnormalities in these seven metabolic pathways jointly affect the Qi-Yin deficiency pattern of dry eye.

4.2 Metabolic characteristics and TCM phenotypes in dry eye

The heat stagnation in liver meridian pattern of dry eye is a classification in the syndrome differentiation of TCM. TCM theory holds that it is related to the dysfunction of liver function, leading to the stagnation of Qi and the generation of internal heat, causing several eye symptoms, such as dry eyes, red eyes, blurred vision, and emotional fluctuations. Heat stagnation refers to the accumulation of Qi stagnation and heat evil in the body, leading to liver Qi discomfort and poor Qi movement. Heat stagnation in liver meridian is linked to psychological states such as emotional depression, mental tension, and anger, which are associated with the regulation of hormones and other biologically active substance levels, potentially influencing the endocrine system and metabolic status.

In this study, the caffeine and purine metabolism pathways were not only significantly enriched in the heat stagnation in liver meridian but also the Qi-Yin deficiency patterns of dry eye, indicating that they may play an important part in the common pathological process of dry eye. Caffeine and purine metabolism belong to one of the energy metabolisms, and their occurrence and development are strongly correlated with free radicals [17]. Caffeine metabolism involves the degradation and clearance process of caffeine, and its metabolites may be closely related to the generation of free radicals, affecting the cellular oxidative stress response as a result. Oxidative stress is a key pathological feature of dry eye, and excessive free radicals can cause damage and inflammatory reactions in ocular tissues. Purine metabolism is involved in the synthesis and utilization of cellular energy, such as the generation of adenosine triphosphate (ATP) and guanosine triphosphate (GTP). Its abnormalities may lead to insufficient cellular energy supply, especially for the high metabolic demand of the lacrimal and meibomian glands, thus exacerbating dry eye symptoms [18]. Unlike the Qi-Yin deficiency pattern of dry eye, an important metabolic pathway in patients with the heat stagnation in liver meridian pattern of dry eye is the biosynthesis pathway of valine, leucine, and isoleucine. Valine, leucine, and isoleucine are all branched-chain amino acids, which are key amino acids that play an important role in muscle protein synthesis, immune function regulation, and liver metabolism. Abnormalities in the metabolism of

branched-chain amino acids are often related to liver dysfunction, which may result in changes in mood and mental state, changes that are similar to the "heat stagnation" described by TCM [19].

Furthermore, long-term nutritional imbalance, including abnormalities in branched-chain amino acids, may cause metabolic disorders in the body, which can affect a person's physical and mental health in various forms (e.g., hormonal imbalance), corresponding to the "liver depression" described by TCM [20]. The Treatise of the True Doctrine of the Golden Coffer in the *Inner Cannon of Haungdi on Plain Questions* (*Huang Di Nei Jing · Su Wen*,《黄帝内经·素问》) recorded that "the liver opens into the eyes." It is believed that a special intrinsic connection between the liver and the eyes exists; thus diseases of the liver are often thought to influence the function of the eyes. Studies have proposed that the liver and eyes share characteristics of co-damage, co-repair, co-material basis, and co-mechanism of action [21]. From the perspective of metabolomics, this further proves the rationality of the classification of dry eye due to stagnant heat in the liver channel.

This study still has some limitations. First, the current sample size is small as an exploratory experiment, which may affect the generalizability of the results. Future research is warranted to determine the minimum required sample size based on the results of this study, using sample size calculation formulas to strengthen the reliability of the findings. Second, the diagnostic criteria for TCM syndromes mainly rely on subjective descriptions, which may impact the reproducibility of the results. Quantitative assessment methods, such as scoring systems or questionnaires, should be introduced and unified diagnostic criteria and procedures should be established in subsequent large-scale studies to reduce the interference of subjective factors. Moreover, the present study did not provide details of the relevant clinical and laboratory testing indicators, which limits the comprehensiveness of dry eye diagnoses. Finally, key metabolites and functional clusters still require further validation. Despite these shortcomings, this research provides a preliminary reference for future large-scale experiments and lays the foundation for in-depth exploration of the metabolic characteristics of dry eye.

5 Conclusion

The clinical metabolism of dry eye patients with Qi-Yin deficiency and heat stagnation in liver meridian patterns has its own characteristics and is significantly varied. Among them, purine and caffeine metabolism pathways are common metabolic characteristics of both patterns of dry eye. dIMP and 2-(formamido)-N1-(5-phospho-D-ribosyl) acetamidine can be used as biomarkers. In the Qi-Yin deficiency pattern, the significant increase of metabolic pathways, such as lysine degradation, ovarian steroidogenesis, cholesterol metabolism, pyrimidine metabolism, and bile secretion, remains the main feature. The dry eye of the heat stagnation in liver meridian pattern is featured with the inhibition of the valine, leucine, and isoleucine biosynthesis pathway. The above results prove the rationality of the TCM syndrome differentiation classification and are of crucial reference for the exploration of the pathogenesis of dry eye and the development of related drugs.

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Competing interests

The authors declare no conflict of interest.

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基于泪液代谢组学研究气阴两虚型与肝经郁热型干眼的代谢特征

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【摘要】目的 探究肝经郁热型和气阴两虚型干眼患者的代谢差异，明确两者的代谢特征。**方法** 选取2020年10月1日至2021年10月30日于湖南中医药大学第一附属医院眼科病房及门诊就诊的干眼患者为研究对象，依据中医证型分为肝经郁热型组和气阴两虚型组；按照随机数字表法纳入健康管理科体检的健康志愿者为健康组。采用高分辨率液相色谱-质谱技术（LC-MS）分别对肝经郁热型和气阴两虚型干眼患者及健康志愿者泪液进行检测，通过多元统计分析方法筛选出差异代谢物，并对差异的代谢物进行京都基因与基因组百科全书（KEGG）通路富集，最后对差异蛋白和代谢物进行关联分析，以验证和补充代谢物。**结果** 研究最终共纳入干眼症患者32例，其中肝经郁热型16例，气阴两虚型16例；14名健康志愿者作为健康组。三组患者基线特征比较差异无统计学意义（$P>0.05$）。气阴两虚型组共确认了412种标志物，主要包括脂质及脂质样分子、有机酸及其衍生物、有机杂环化合物、核苷、核苷酸和类似物；肝经郁热型组共确认了112种代谢物，主要包括有机酸及衍生物和脂质及脂质样分子；结合KEGG通路富集结果和差异标志物的相对含量分析可得，嘌呤代谢及咖啡因代谢途径是所有干眼的共有代谢特征。其中脱氧肌苷酸（dIMP）和2-甲酰胺基-N1-（5-磷酸-D-核糖基）乙脒可作为其生物标志物。而气阴两虚型中以赖氨酸降解、卵巢类固醇生成、胆固醇代谢、嘧啶代谢和胆汁的分泌等代谢途径的显著增强为主要特征（$P<0.05$）。肝经郁热型干眼以缬氨酸、亮氨酸和异亮氨酸途径的抑制为主要特征（$P<0.05$）。**结论** 代谢组学可以作为中医证型分类的有效依据，不同证型干眼症在代谢物的种类和含量上均具有典型的特征，且与中医的证候分型的表型特征相对应。本研究初步证实了中医证型分类的合理性，并对干眼病机制和药物开发提供了重要参考。

【关键词】气阴两虚；肝经郁热；干眼；泪液；代谢组学