

Metabolomics analysis revealed acupuncture treatment target the upstream for dry eye disease

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Abstract

• **AIM:** To find out the pathogenesis of dry eye disease (DED) and the possible mechanisms of available effective treatments.

• **METHODS:** A non-targeted technology, ultra-high performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS), to investigate metabolic characterizations for tear samples from 18 patients with DED upon drug or acupuncture treatment.

• **RESULTS:** A total of 190 named metabolites were identified, which presented so far the broadest tear metabolome. Further analysis indicated a significantly distinct metabolomics profile among all patients, but very subtle metabolic differences upon drug or acupuncture treatment. On one hand, only six significantly changed metabolites were determined after drug treatment,

five of which including inosine, monopalmitin, urate, propionylcarnitine, and nicotinamide were all increased and involved in inflammatory responses. On the other hand, merely four metabolites including alanine, serine, and homoserine were found to be significantly different. Further pathway analysis of those six and four significantly changed metabolites revealed that only one pathway, aminoacyl-tRNA biosynthesis was significantly influenced in acupuncture-treated patients, which were highly associated with the cause of the disease. The results here indicated acupuncture treatment may address the cause rather than the symptoms for dry eye disease, displaying partially better compared with drug treatment.

• **CONCLUSION:** Collectively, this work extended our understanding on the key regulatory elements or pathways involved in the potential mechanisms of available effective treatments, and would be useful for providing novel potential targets and therapeutic strategies for DED.

• **KEYWORDS:** artificial tears; acupuncture; dry eye; metabolomics; tobramycin dexamethasone eye drops

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INTRODUCTION

Dry eye disease (DED), also called keratoconjunctivitis sicca, is a chronic multifactorial disorder of the tears and ocular surface resulting in symptoms of ocular discomfort and visual disturbance^[1]. The most common symptoms behind DED include dryness, redness, burning/stinging, photophobia, ocular fatigue and blurred vision. Unfortunately, DED is increasing in prevalence affecting about 5%-30% of the worldwide population of different ages, which can significantly impair quality of life, as well as the patients' economic burden^[2]. Moreover, the diagnosis of DED is supposed to be difficult due to poor correlations between reported symptoms and available clinical tests^[3].

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The treatment of patients with DED evolves continuously and quite a number of treatment strategies are now available, including artificial tears, alternative anti-inflammatory drugs, immunomodulators, oral supplements, hormonal therapy, and acupuncture^[4]. Among them, tear replacement by artificial tears is currently the most widely used therapy for DED. As reported, artificial tears can lower tear osmolarity and dilute proinflammatory factors on the ocular surface, and also smooth the corneal surface to improved vision^[5]. However, artificial tears may finally lose their efficacy over time since they do not target the underlying causes of DED. Anti-inflammatory drugs represents a necessary step during the chronic therapy of DED, and especially non-steroidal anti-inflammatory drugs (NSAIDs) are demonstrated as prospective therapy for DED^[2]. Although these drugs target the underlying etiology and reduce inflammation and eye pain for patients with DED, they could reduce their eye sensitivities at the same time. Moreover, oral supplements of omega3 (ω 3) fatty acids have been recommended in the treatment of moderate DED, which promote tear film stabilization and play a beneficial role in DED^[6]. Additionally, acupuncture therapy is also determined to be effective for dry eye patients without serious side effects. Recent studies showed acupuncture could improve the signs and symptoms for patients with DED after 4wk of treatment^[7]. Recently, the advent of the omics era (especially proteomics and metabolomics) has dramatically increased our understanding on the pathogenic mechanisms of complex diseases, as well as the potential mechanisms of improvement after various treatments^[8-9]. For example, proteomics shows great potential application in large-scale clinical ophthalmology studies, which allows the simultaneous study of thousands of proteins and/or peptides in biological samples^[10-11]. Proteomic analysis of 29 human aqueous humor samples from patients with cataract and glaucoma with and without the pseudoexfoliation syndrome revealed no significant difference between glaucoma and cataract AH proteomes, while apolipoprotein D was found to be a putative biomarker for pseudoexfoliation syndrome^[10]. Moreover, Liu *et al*^[12] employed two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry (2D nano-LC-MS/MS) to determine whether acupuncture is effective at treating dry eye disease and to find out the possible mechanisms. The results revealed that an acupuncture plus artificial tears (AC+AT) treatment improved clinical symptoms and increased protein synthesis and secretion. Metabolomics has emerged as a powerful tool, allowing simultaneous analysis of hundreds of metabolites in one biological sample. It has great potential to screen out biomarkers and functional pathways for various diseases including eye diseases, and thus lead to novel therapies^[3,9,13]. We previously used gas

chromatography coupled to time-of-flight mass spectrometer (GC/TOF MS) for metabolites profiling of 40 aqueous humor samples from patients with high myopia and the controls, which revealed that 29 significantly changed metabolites and their regulatory aspects of metabolic pathways might be key regulatory elements or pathways in the development of high myopia. Furthermore, Galbis-Estrada *et al*^[14] applied nuclear magnetic resonance spectroscopy of hydrogen-1 nuclei (¹H NMR S) to investigate the role of the oral supplements with antioxidants and omega 3 fatty acids in relation to dry eye. The data suggested that the metabolic changes in dry eye syndrome could be modified with appropriate oral supplementation with antioxidants and essential polyunsaturated fatty acids.

Tear fluid is a valuable source for objective analysis on various eye diseases, whose components have various roles including protecting and maintaining the ocular surface^[15]. It is composed of proteins, carbohydrates, lipids, electrolytes, and some small organic molecules, while defective quality and quantity of tear could lead to dry eye. In this study, we took advantage of a non-targeted technology, ultra-high performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS), to report the most comprehensive human tear metabolome from dry eye patients up to date. And more importantly, the roles of drug and acupuncture treatment for dry eye disease were further investigated, respectively.

SUBJECTS AND METHODS

Ethical Approval All Participants provided written informed consent and the present study was approved by the Ethics Committee of the Third Affiliated Hospital of Beijing University of Traditional Chinese Medicine. Meanwhile, the study was performed in accordance with the Declaration of Helsinki.

Subjects Eighteen patients with DED were included in the present study (Table 1). The ophthalmic examination included subjective symptoms, visual acuity, schirmer tear I test (SIT) with anesthesia (basis secretory test, BST), and tear break-up time (BUT). Each patient was asked for subjective symptoms such as burning, itching, foreign body sensation, dryness, and photophobia. Patients were classified as having DED if they had the above-mentioned symptoms and abnormalities of test dynamics determined by a SIT score less than 10 mm/5min or a BUT score less than 10s. Notably, patients with neither Sjögren syndrome nor aqueous-deficient dry eye were excluded from this study. The tears were collected by capillary method. Each time we collected 5-15 uL tears by 25 μ L capillary tube from the canthus, and immediately transferred them into the EP tube and stored at -80 C refrigerator until further analysis.

Treatment Procedure All participants were divided into either drug treatment group or acupuncture treatment group,

while both lasted for 3wk. In the drug treatment group, each patient was offered with artificial tears and tobramycin dexamethasone eye drops. The two drugs were successively dropped into the conjunctival sac with a minimal interval of 10min apart, one drop each time and four times 1d. While the acupuncture treatment was taken by the same ophthalmologist, which was mainly consisted of the acupoints of Jingming, Chengqi, Shangming, Cuanzhu, Hegu. Another different acupoints were further allocated according to the differentiation of symptoms and signs: For type of fei-yin deficiency with Kongzui and Chize acupoints; for type of liver-yin and kidney-yin deficiency with Taixi and Sanyinjiao acupoints; for type of damp heat in the spleen and stomach with Quchi and Zusanli acupoints. Keep the needles for 30min after getting air. Both eyes of each patient were treated every other day and totally ten times were performed in one course lasting for 3wk. After treatment, their symptoms, SIT and BUT scores were recorded again, and tears were also collected. According to the paired *t*-test (SPSS 17.0 software), no significantly differences were identified in BUT & SIT before treatment between those two groups. Likewise, no significantly differences of IOPs between all enrolled patients before and after treatment were observed.

Metabolomics Analysis The metabolites profiling of all tear samples was conducted as our previous report^[16]. Briefly, 300 μ L methanol was firstly added into each tear sample (about 15 μ L), which was then ultrasonicated for 20min and centrifuged at 12 000 \times g in 4°C for 10min. 250 μ L of the supernatant was transferred into 1.5 mL centrifuge tube and completely dried in a vacuum concentrator completely. Secondly, 75 μ L methanol were added and then centrifuged at 12 000 \times g in 4°C for 10min. 50 μ L of the supernatant was finally transferred into a glass vial for metabolomic analysis. A volume of 3 μ L was injected into the Agilent 1290 Infinity II LC TM system with an Agilent Eclipse-plus C18 column (150 \times 3.0 mm i.d., 1.8 μ m). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (100% acetonitrile), while the flow rate was 0.40 mL/min. Gradient elution conditions were set as follows: 0min, 98.0% A; 1.0min, 98% A; 5.0min, 60% A; 12.0min, 30% A; 15.0min, 5% A; 20.0min, 5% A. MS detection was conducted on the Agilent 6550 iFunnel/Q-TOF mass spectrometer with Agilent Jet-Stream source^[16]. Metabolites annotation was finished by searching Personal Compound Database and Library (PCD/PCDL), Massbank database and literatures^[14,17-19]. Moreover, the softwares of MassHunter Acquisition 6.0, MassHunter Qualitative 6.0 and Mass Profinder 6.0 were respectively used for data acquisition, data review and metabolite identification, peak area extraction.

Data Analysis Metabolomics data were firstly normalized as our previous reports, which registered the median level of

Table 1 Information of human tear samples from dry eye patients

Groups	Sample name		Age (y)	Gender
	Before	After		
Acupuncture treatment group	bAT1	aAT1	47	F
	bAT2	aAT2	81	F
	bAT3	aAT3	63	F
	bAT4	aAT4	42	M
	bAT5	aAT5	64	M
	bAT6	aAT6	76	F
	bAT7	aAT7	55	M
	bAT8	aAT8	45	M
	bAT9	aAT9	64	F
Drug treatment group	bDT1	aDT1	76	F
	bDT2	aDT2	78	F
	bDT3	aDT3	68	M
	bDT4	aDT4	48	M
	bDT5	aDT5	56	F
	bDT6	aDT6	60	M
	bDT7	aDT7	59	F
	bDT8	aDT8	74	M
	bDT9	aDT9	48	M

M: Male; F: Female.

each metabolites to equal to one (1.00) while missing values (if any) were imputed with the observed minimum ones^[7]. Mev (MultiExperiment Viewer) 4.8 software was further used for hierarchical cluster analysis, and meanwhile SIMCA-P software (v13.0, Umetrics, Malmö, Sweden) was applied for both principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Moreover, paired *t*-test (SPSS 17.0 software) was used to identify metabolites which were significantly different between before and after treatment in patients with DED. Metabolites with both VIP (variable importance in the projection) values in PLS-DA model more than 1 and *P* values (in *t*-tests) less than 0.05 were considered significant. In addition, metabolic pathway analysis of significantly different metabolites was conducted by MetaboAnalyst web server (<http://www.metaboanalyst.ca>). Significant pathways were judged based on *P* threshold 0.05.

RESULTS

Clinical Characteristics of Dry Eye Patients In the present study, we recruited 18 patients with DED and their clinical characteristics were listed in Table 1. Nine patients were selected into drug treatment group, while the rest 9 patients were enrolled into acupuncture treatment group. The median age of the patients in drug treatment group was 63-year-old (range, 48-76y), 4/9 of which were female. On the other hand, the average age of the patients treated by acupuncture treatment group was 59.7-year-old (range, 42-81y), and

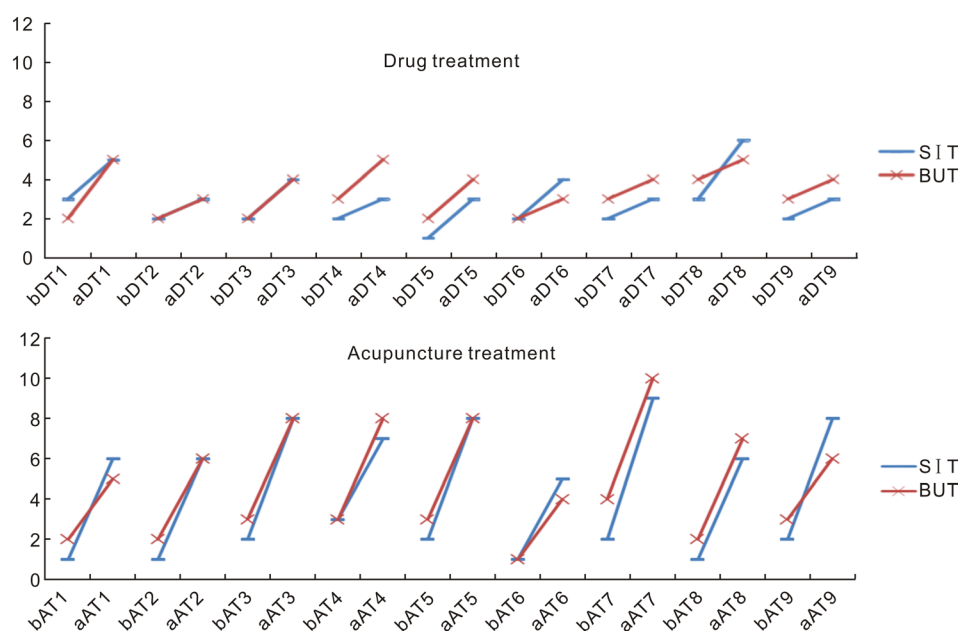


Figure 1 The information of the SIT and BUT scores record in both drug and acupuncture treatment for patients with DED.

meanwhile 5/9 of these patients were female. The statistical analysis showed that there were no significantly difference of between the two groups for both sex and age. Moreover, both scores of SIT and BUT for these 18 patients before and after treatment were also recorded (Figure 1). Obviously, scores of SIT and BUT of these 18 patients were both low at the beginning but significantly improved after treatment. Notably, the changes of SIT and BUT scores for patients in acupuncture treatment group were larger than those in drug treatment group. The results here indicated that both acupuncture and drug treatments were effective for patients with DED, and especially acupuncture treatment seemed to be partially better compared with drug treatment.

Metabolic Characterization of Tears from Dry Eye Patients In order to comprehensively uncover the human tear metabolome, we here applied UHPLC-Q-TOF-MS to metabolic profiling of 36 tears samples from 18 dry eye patients. A total of 190 named metabolites were identified in the tested tear samples. Those identified 190 metabolites were categorized into 13 groups according to the database from Kyoto Encyclopedia of Genes and Genomes (KEGG). The largest group contained 36.8% of the total number of detected metabolites, which were 70 amino acids and derivatives. The second largest group (22.1%) was 42 lipids, followed by 24 (12.6%) nucleotides, and 20 (9.5%) carbohydrates.

An un-supervised approach, hierarchical cluster analysis was firstly performed to investigate metabolic variations among the 18 tear samples from dry eye patients before treatment (Figure 2A). The heat map indicated remarkable diversities in metabolite abundance across these dry eye patients. Several metabolites such as 3-(pyrazol-1-yl)-alanine (Figure 2B),

cytidine, cytosine, cyclic AMP, and guanosine 2'3'-cyclic phosphate displayed low abundance in all the samples, while the enrichment of certain metabolites appeared to be individual-specific. For example, pyridoxine was highly abundant only in sample bAT7 when compared to that in other 17 tear samples (Figure 2C). Moreover, the major of the detected metabolites in several tear samples including sample bDT4 and bAT3 seemed to have lower abundance, while most of 190 metabolites displayed highly enriched in sample bAT5. To further provide a snapshot of the information hidden in the metabolomic data, we conducted another un-supervised approach, PCA, for all 36 tear samples from patients with DED before and after treatment. As shown in Figure 3, PCA could not distinguish tear samples with different treatment groups (acupuncture and drug treatment groups), and different groups (untreated and treated groups). However except bAT5 and aAT5 & bDT1 and aDT1, every two tear samples from the same individual displayed close in the PCA score plot. The results confirmed that patients with DED had remarkable metabolic variation, and more importantly either acupuncture or drug treatment had little effect on metabolomics profile.

Metabolic Changes in Dry Eye Patients After Drug Treatment To exactly determine the metabolic differences between patients with DED before and after drug treatment, the supervised statistical method PLS-DA was then employed. Obviously, the tear samples before treatment clustered together, as did those after treatment (Figure 4A). Furthermore, six metabolites (Table 2 and Figure 4B) were determined to be significantly changed ($VIP \geq 1$ and $P \leq 0.05$), playing important roles for the separation. Among them, the levels of 5 compounds were increased, while only glycerate showed lower

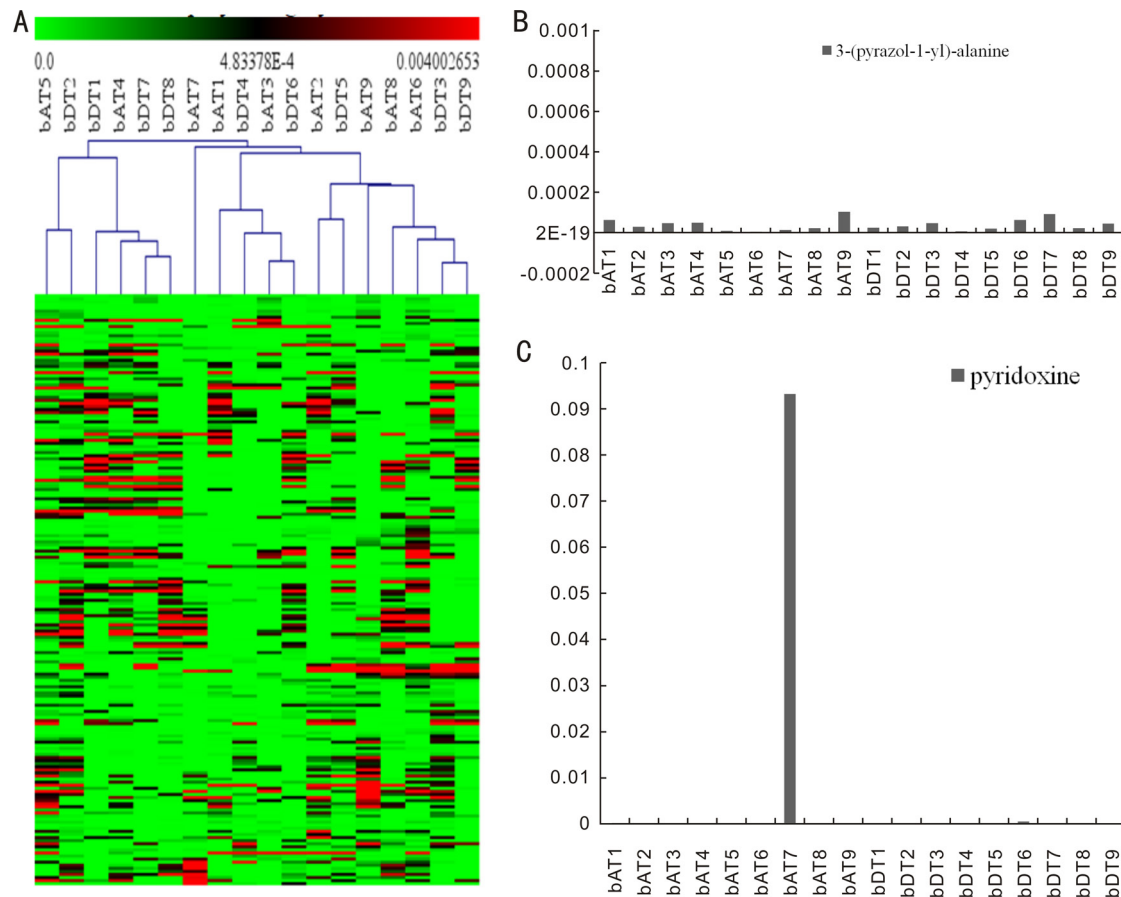


Figure 2 Metabolic variations among the 18 tear samples from dry eye patients before treatment A: Heat map representation of the 190 identified metabolites across all tears samples from 18 dry eye patients before treatment. Each line represented one metabolite. The deeper the red color, the higher its level in the tear samples; while the deeper the green color, the lower its level in the tear samples. B: The levels of 3-(pyrazol-1-yl)-alanine across all tears samples from 18 dry eye patients were shown in greater detail; C: The levels of pyridoxine across all tears samples from 18 dry eye patients were shown in greater detail.

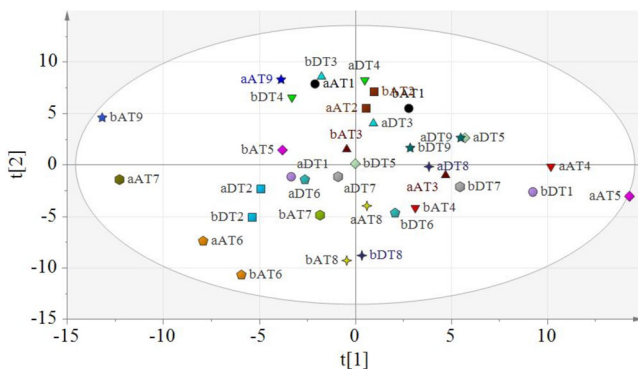


Figure 3 PCA scores plot generated from the 190 identified metabolites across all 36 tears samples from 18 dry eye patients before and after (drug/acupuncture) treatment.

level after treatment. These metabolites were related to five classes such as carbohydrates, organic acids and derivatives, lipids, nucleotides, and cofactors. In addition, further metabolic pathway analysis of these six significantly different metabolites revealed six relevant pathways, among which purine metabolism, and nicotinate & nicotinamide metabolism were significantly influenced ($P < 0.05$, Figure 5A).

Metabolic Changes in Dry Eye Patients After Acupuncture Treatment

Likewise, we also conducted pls-da and paired t -tests to find out the metabolic changes resulted from acupuncture treatment for patients with DED. As shown in Figure 4C, tear samples from patients in the untreated group were clearly separated from those in the treated group. Together with paired t tests, the results showed that only 4 metabolites (Table 3 and Figure 4D) were significantly changed ($VIP \geq 1$ and $P \leq 0.05$), responsible for the separation. Interestingly, all four metabolites displayed lower levels in patients with DED after acupuncture treatment, whose ratios ranged from 0.50 to 0.79. Meanwhile, three of four metabolites belonged to class of amino acids and derivatives, while only one compound cytidine was grouped into class of nucleotides. Additionally, metabolic pathway analysis of these 4 significantly changed metabolites revealed that only one pathway, aminoacyl-tRNA biosynthesis was significantly influenced ($P < 0.05$, Figure 5B).

DISCUSSION

DED is considered to be one of the top three most prevalent chronic eye diseases, which significantly interferes with

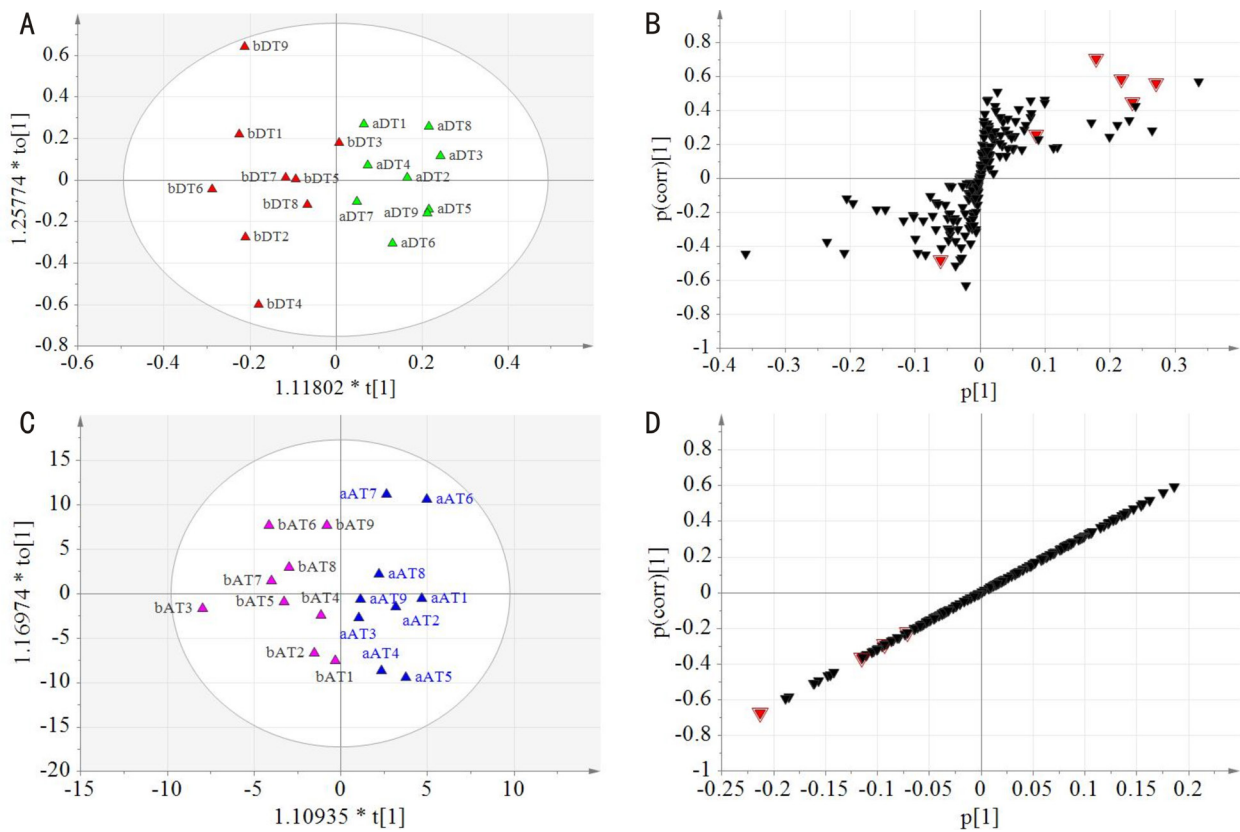


Figure 4 PLS-DA models for dry eye patients before and after treatment A: Score plot in PLS-DA model for dry eye patients before and after drug treatment; B: S plot in PLS-DA model for dry eye patients before and after drug treatment; C: Score plot in PLS-DA model for dry eye patients before and after acupuncture treatment; D: S plot in PLS-DA model for dry eye patients before and after acupuncture treatment. Significantly changed metabolites were labeled with red triangle.

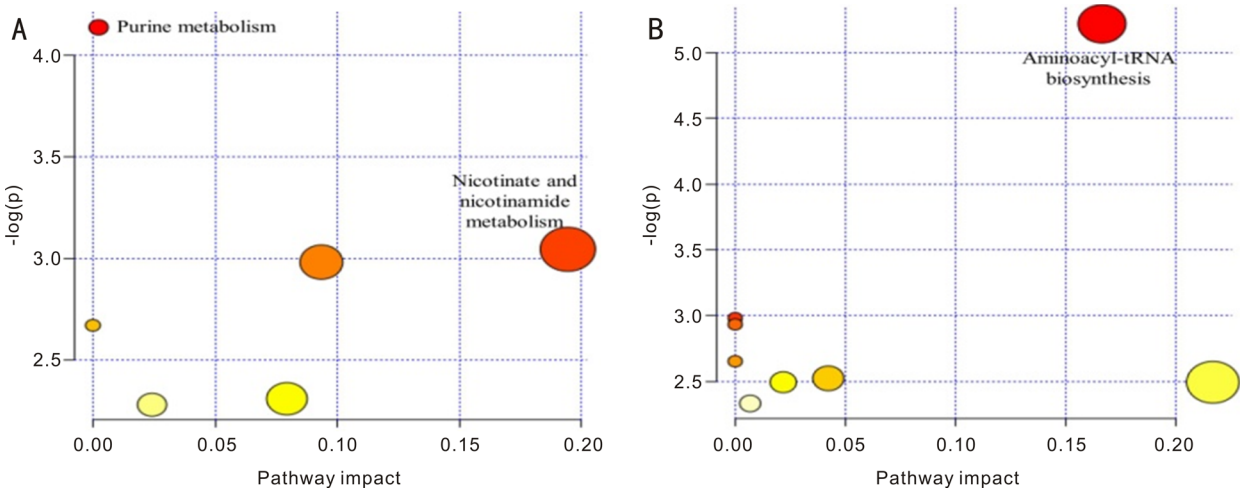


Figure 5 Metabolic pathway analysis A: Metabolic pathway analysis of 6 significantly different metabolites between dry eye patients before and after drug treatment by using MetaboAnalyst web server; B: Metabolic pathway analysis of 4 significantly different metabolites between dry eye patients before and after acupuncture treatment by using MetaboAnalyst web server.

patients' well-being and increases their economic burden of healthcare costs^[4]. However, it is difficult to diagnose and treat due to the lack of correlation between the signs and symptoms of DED^[20]. As a result, it is very important to extend our understanding on the pathogenesis of DED and the possible mechanisms of available effective treatments. Here we used the

non-targeted technology, UHPLC-Q-TOF-MS to investigate the metabolic differences between dry eye patients before and after (drug/acupuncture) treatment. This study may not only provide new insight into the metabolomics features in patients with DED, but present potential targets therapy and therapeutic strategies as well.

Table 2 List of six significantly different metabolites between dry eye patients before and after drug treatment

Superclass	Compound name	HMDB number	KEGG number	Fold change (After/Before)	P
Carbohydrates	glycerate	HMDB00139	C00258	0.60	0.03
Organic acids and derivatives	urate	HMDB00289	C00366	1.48	0.02
	monopalmitin	HMDB31074	-	2.61	0.00
Lipids	propionylcarnitine	HMDB00824	C03017	1.31	0.02
Nucleotides	inosine	HMDB00195	C00294	1.39	0.04
Cofactors	nicotinamide	HMDB01406	C00153	1.40	0.01

Table 3 List of four significantly different metabolites between dry eye patients before and after acupuncture treatment

Superclass	Compound name	HMDB number	KEGG number	Fold change (After/Before)	P
Amino acids and derivatives	alanine	HMDB00161	C00041	0.70	0.02
-	serine	HMDB00187	C00065	0.59	0.03
-	homoserine	HMDB00719	C00263	0.79	0.04
Nucleotides	cytidine	HMDB00089	C00475	0.50	0.04

Generally, the main analytical platforms for metabolomics studies are NMR, GC-MS, and LC-MS. Each analytical platform has its own unique advantages and disadvantages, for instance, NMR is efficient, highly reproducible, and non-destructive, but it has relatively lower sensitivity^[9]. GC-MS is the most widespread tandem technique and has high sensitivity and specificity, but especially targeting for volatile non-polar compounds. LC-MS is also an important metabolomic technology, suitable for analyzing large highly polar metabolites. UHPLC, evolved from LC, has greater analytical power, which allows high throughput sample handling and down scaling of sample volume^[21]. Since tear is mainly composed of non-volatile compounds including amino acid and lipids, we thus employed UHPLC-Q-TOF-MS global metabolomics analysis on 38 tear samples from patients with DED. Totally 190 named metabolites were identified in the tear, most of which were amino acids & derivatives, and lipids. These identified metabolites were grouped into 13 classes, covering most of the central metabolism pathways. It is believed that our study presented so far the broadest tear metabolome especially for dry eye, corroborating the power of the non-targeted and high-throughput technology in uncovering their metabolic characteristics^[14,17-19].

Further analysis indicated a significantly distinct metabolomics profile among all patients with DED, but very subtle metabolic differences upon acupuncture or drug treatment for each individual patient. In the drug treatment group, dry eye patients were offered with artificial tears and tobramycin dexamethasone eye drops. Tobramycin dexamethasone eye drops have been used in ophthalmology since 1983 and

are well-accepted ophthalmic solutions effectively used to prevent infection and control inflammation^[22]. Obviously, the improvement of both SIT and TBUT scores suggested that artificial tears and tobramycin dexamethasone eye drops had an effective and beneficial effect on DED as previously reported^[23]. Notably, metabolomic study showed that there were only six significantly changed metabolites. Of particular interest, all the five increased compounds including inosine, monopalmitin, urate, and propionylcarnitine exert a wide variety of anti-inflammatory responses^[24-27]. For example, inosine was reported to suppress immune responses and protect mice from experimental autoimmune encephalomyelitis, while monopalmitin may be associated with the cellular toxicity in common across various types of autoimmune inflammatory disorders of the central nervous system^[26,28]. Moreover, urate is a powerful antioxidant, which was associated with inflammation, endothelial and renal dysfunction, and cardiovascular disease^[24]. Especially, nicotinamide has been proposed as a therapeutic alternative for the prevention of diseases with metabolic dysfunctions, playing critical roles in regulating the inflammatory response and the expression of genes related to oxidative stress^[29].

Acupuncture has been used as one of the major therapeutic modalities to treat various diseases for several thousands of years in eastern Asian countries^[7]. Recent ophthalmologic studies have shown that acupuncture therapy is an effective treatment for several eye diseases including amblyopia and glaucoma. Especially, the efficacy and effectiveness of acupuncture for DED has also been identified, showing partially better when compared to artificial tear treatment^[30].

The results in our study confirmed that acupuncture treatment was likely better compared with drug treatment according to the scores of SIT and TBUT. Meanwhile, further metabolomic analysis revealed that merely 4 metabolites were significantly changed in patients after acupuncture treatment, three of which including alanine, serine, and homoserine belonged to amino acids and derivatives. Metabolic pathway analysis of these 4 significantly changed metabolites indicated that only one pathway, aminoacyl-tRNA biosynthesis was significantly influenced. Interestingly, our previous study revealed that the activity of amino acids metabolism was increased in tear samples from patients with DED when compared with those from normal individuals^[31]. As a matter of fact, amino acids metabolism plays important roles in multiple aspects of cell biology, which have been identified in various eye diseases including high myopia and cataract^[9,32]. Additionally, further metabolic pathway analysis of those 34 significantly changed metabolites in our previous study determined that four pathways including aminoacyl-tRNA biosynthesis were significantly enriched, which was also found in dry eye patients treated by acupuncture^[31]. The above changed amino acid associated metabolites and pathways (especially aminoacyl-tRNA biosynthesis) may be involve in protein synthesis and secretion and thus play significant roles on the effective mechanisms of acupuncture therapy^[12].

In conclusion, we for the first time comprehensively uncovered metabolic characteristics on dry eye and observed a remarkable metabolic variation among all patients with DED. Moreover, either drug or acupuncture treatment had little effect on metabolomics profile. Importantly, acupuncture may have a distinctive mechanism of improvement on dry eye, which displayed to be partially better compared with drug treatment. The results here enabled us to extend our understanding on the key regulatory elements or pathways involved in the underlying mechanisms of DED treatment, and may further provide novel potential targets and therapeutic strategies. Since the number and types of samples were limited, extensive validation of our findings would be further performed in a larger scale study^[33].

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