Original Article
Identification of diagnostic markers related to fecal and plasma metabolism in primary Sjögren’s syndrome

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Abstract: Background: Accurate diagnostic techniques for patients with primary Sjögren’s syndrome (pSS) are needed. This study aimed to investigate new biomarkers related to fecal and plasma metabolism from pSS patients.

Methods: The feces and plasma of 21 pSS patients and 18 controls admitted to the Second Hospital of Shanxi Medical University were collected for analysis. Metabolites in feces and plasma were quantified using liquid chromatography-mass spectrometry. The metabolic pathway alterations caused by pSS were studied and the expression of metabolites in the intersecting pathway was analyzed in the feces and plasma of pSS patients. Metabolites that showed the same alterations in feces and plasma in pSS patients were considered as diagnostic markers and receiver operating characteristic curves were generated to analyze the sensitivity of these markers in diagnosing pSS.

Results: There were 114 and 92 upregulated metabolites and 54 and 125 downregulated metabolites in the feces and plasma of pSS patients, respectively. These metabolites were enriched in 8 pathways for feces and 12 pathways for plasma. Arginine biosynthesis, Linoleic acid metabolism, Tyrosine metabolism, Taurine and hypotaurine metabolism were pathways enriched by metabolites in both samples. Twelves metabolites were enriched in the above four pathways, while only 9,10-Diepoxyoctadecanoate, Tyramine, 9-OxoODE and 2-Hydroxyethanesulfonate showed the same trend. The candidate diagnostic markers were all predictive, with better diagnostic sensitivity in plasma samples.

Conclusions: 9,10-12,13-Diepoxyoctadecanoate, Tyramine, 9-OxoODE, 2-Hydroxyethanesulfonate were metabolism-related diagnostic markers for pSS feces and plasma.

Keywords: Primary Sjögren’s syndrome, metabolomics, feces, plasma, liquid chromatography-mass spectrometry

Introduction

Sjögren syndrome (SS) is an autoimmune disease leading to secretory gland dysfunction with an incidence of 0.2-0.5% in the adult population, which contributes to dryness of major mucosal surfaces, such as the mouth, eyes, nose, pharynx, larynx and vagina [1, 2]. Treatments for SS include topical and systemic treatments for the sicca and systemic symptoms of disease [3]. This condition occurs in isolation or in correlation with organ-specific autoimmune diseases, such as thyroiditis, primary biliary cirrhosis or cholangitis, when it is termed as primary SS (pSS) [4]. A number of genetic and environmental factors may intertwine in its etiology, including being female (in a 9/1 ratio) and ethnicity [5]. Given the nature of the available diagnostic tools, there is increasing agreement that novel biomarkers for early diagnosis pSS are urgently needed [6, 7].

It has been revealed that metabolites have regulatory roles in the function of the immune system [8]. The term “metabolomics” was first used at the beginning of this millennium to recognize functional genomics devoted to the analysis of metabolites [9]. Therefore, global or untargeted metabolomics is an attractive method to improve the understanding of an organism’s response to normal and abnormal biological processes and external stimuli [10]. Liquid
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Chromatography-mass spectrometry (LC-MS) has been utilized to determine biomarkers and therapeutic targets by examining the salivary, tear, urinary and plasma metabolomics [11-13]. Interestingly, fecal metabolomics has been applied to screen potential biomarkers for systemic lupus erythematosus, another autoimmune disease [14]. The human gut microbiome fundamentally is a metabolic organ which is imperative to the digestion of food and the chemical by-products resulting from gut microbiome digestion play significant roles in human metabolism, health, and disease [15]. Even though severe intestinal dysbiosis has been found prevalent in pSS and is related to clinical and laboratory markers of systemic disease activity [16], the role of fecal metabolomics in pSS remains largely unexplored. Therefore, the main goal of the present study was to characterize plasma and fecal metabolomics in pSS patients and to explore possible marker candidates. We compared both fecal and plasma metabolomics of pSS patients and healthy controls and sought to investigate if LC-MS-derived fecal and plasma metabolomics could reveal a signature of pSS.

Materials and methods

Ethics statement

The study was permitted by the Ethic committee of the Second Hospital of Shanxi Medical University as per the Declaration of Helsinki. All participants provided written informed consent.

Study populations

Twenty-one pSS and 18 controls who received health examination at the Second Hospital of Shanxi Medical University from December 2019 to March 2021 were included. All patients met the pSS classification criteria defined by American College of Rheumatology and European League against Rheumatism (EULAR) [17]. Inclusion criteria (at least one of the following was positive): (1) daily troublesome dry eyes for more than 3 months; (2) recurrent gritty sensation in the eyes; (3) need for artificial tears 3 or more times daily; (4) a daily wetting of a dry mouth for more than 3 months; (5) frequently drink liquids to aid in swallowing dry food, or (6) suspicion of SS from the EULAR primary Sjögren’s syndrome disease activity (ESSDAI) questionnaire (at least one domain with positive item) [18]. Exclusion criteria: (1) history of head and neck radiation therapy; (2) active hepatitis C virus infection; (3) AIDS; (4) sarcoidosis; (5) amyloidosis; (6) graft-versus-host disease; or (7) IgG4-associated disease. The classification of pSS was applied to any individual who met the inclusion criteria, did not have any condition described in the exclusion criteria, and who had a score ≥ 4 when summing the weights from the following items: (1) focal lymphocytic infiltration of the lacrimal gland with a focal index of ≥ 1 foci/4 mm² for a score of 3; (2) positive serum anti-Sjögren’s syndrome antigen A antibody for a score of 3; (3) ocular staining score ≥ 5 or van Bijsterveld score ≥ 4 on at least one eye for a score of 1; (4) Schirmer test ≤ 5 mm/5 min on at least one eye was scored as 1; and (5) unstimulated whole salivary flow rate ≤ 0.1 mL/min [19] for a score of 1. Cholinergic drugs should be discontinued before performing the above 3, 4 and 5 tests. To avoid additional effects of gut microbes on intestinal metabolites, all study participants had not taken probiotics or antibiotics within 1 month prior to the study.

Sample preparation

Feces from patients and healthy controls were placed in -80°C freezer and were thawed on ice at the time of use (one feces sample was excluded due to insufficient volume). All samples were melted at 4°C, and 100 mg (± 1%) of samples were accurately weighed and transferred into 2 mL EP tubes, followed by the addition of 0.6 mL 2-chlorophenylalanine (4 ppm) methanol (20°C). The samples were vortexed for 30 s. Following the supplementation of 100 mg glass beads, the samples were transferred to a tissue grinder, ground at 55 Hz for 60 s, sonicated at room temperature for 10 min, and centrifuged at 12,000 rpm at 4°C. The supernatant was filtered via a 0.22-μm membrane for 10 min, and the filtrate was added to the assay bottle for LC-MS detection. Each sample to be tested (20 μL) was mixed into quality control (QC) samples.

Plasma from patients and healthy controls was placed in -80°C freezer and was later thawed on ice for experimentation. Each sample (100 μL) was transferred into a centrifuge tube (2 mL), and supplemented with 400 μL of metha-
nol (-20°C). The samples were vortexed for 1 min, centrifuged at 12,000 rpm for 10 min at 4°C. The obtained supernatant was then placed into a fresh centrifuge tube (2 mL). After drying, the residue was reconstituted in 150 µL 2-chlorophenylalanine (4 ppm) dissolved in 80% methanol solution. The obtained supernatant was filtered using a 0.22 µm membrane. Each sample to be tested (20 µL) was mixed into QC samples.

**LC-MS detection**

ACQUITY UPLC® HSS T3 (2.1 × 150 mm, 1.8 µm) was applied for chromatographic separation (column temperature: 40°C). The mobile phase was comprised of positive ion 0.1% formic acid water (C) - 0.1% formic acid acetonitrile (D); negative ion 5 mM ammonium formic acid water (A) - acetonitrile (B). The gradient program was as follows: 0-1 min at 2% B/D, 1-9 min at 2%-50% B/D, 9-12 min at 50%-98% B/D, 12-13.5 min at 98% B/D, 13.5-14 min at 98%-2% B/D, and 14-20 min, 2% D-positive mode (14-17 min at 2% B-negative mode). The gradient was operated at 0.25 mL/min (injection volume 2 µL).

The electrospray ionization (ESI) source operated successively in negative and positive ionization modes. Voltages were set at 2.50 kV and 3.50 kV (negative and positive ionization modes respectively). Rest parameters were as follows: Capillary temperature of 325°C, sheath gas of 30 arb, and auxiliary gas of 10 arb. During the full-scan acquisition (81-1000), the instrument operated at a resolution of 70,000. Secondary cleavage was carried out using HCD with a collision voltage of 30 eV while removing unnecessary MS/MS information using dynamic exclusion.

**Data pre-processing**

The raw data was converted into mzXML format (xcms input file format) by Proteowizard software (version 3.0.8789). After peak identification, filtration, and alignment using XCMS package in R program (version 3.3.2) (bw = 5, ppm = 15, peak width = c (5,30), mzwid = 0.015, mzdif = 0.01, method = “centWave”), a data matrix including mass to charge ratio (m/z), retention time and intensity was obtained. There were 4,298 precursor molecules in positive ion mode and 3,552 in the negative ion mode and the data were loaded onto Excel. Batch normalization was carried out on the data for intensity.

**Multivariate statistical analyses**

SIMCA-P (v13.0) and the R language ropls package [20] were applied for multivariate statistical analysis. Autoscaling, Mean-centering and scaled to unit variance was applied. The multivariate statistical analysis included principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), as well as orthogonal partial least squares discriminant analysis (OPLS-DA).

**Metabolite identification and analysis of metabolic pathways**

The identification of metabolites was started by confirming the exact molecular weight of the metabolites (molecular weight error < 30 ppm). Annotation was performed by using in HumanMetabolome Database (http://www.hmdb.ca), Metlin (http://metlin.scripps.edu), massbank (http://metlin.scripps.jp/), LipidMaps (http://www.hmdb.ca), and mzclound (https://www.mzcloud.org) and matched with fragmentation information obtained from MS/MS experiments to obtain accurate metabolite information.

MetPA, a part of metaboanalyst (www.metaboanalyst.ca), is primarily based on the KEGG metabolic pathway. The metabolic pathways associated with the two groups were analyzed using the hypergeometric test and the pathway topology was the Relative-betweenness Centrality.

**Relative operating characteristic (ROC) curves**

ROC analysis was performed to obtain area under curve (AUC) using the R package pROC (version 1.17.0.1). Information on the relative expression of metabolism-related diagnostic markers and the source of the samples (whether they were pSS patients or not) was obtained. ROC analysis for diagnostic discrimination was performed and AUC and confidence intervals were evaluated.

**Statistics**

All analyses were made using GraphPad Prism 8.0.2 (GraphPad, San Diego, CA, USA). Values
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**Table 1. Baseline characteristics of pSS patients and healthy controls**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>pSS patients (n = 21)</th>
<th>Healthy control (n = 18)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Age (year)</td>
<td>56.42 ± 12.41</td>
<td>54.5 ± 8.7</td>
<td>0.599</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>62.71 ± 10.63</td>
<td>63.29 ± 11.2</td>
<td>0.869</td>
</tr>
<tr>
<td>ESSDAI</td>
<td>10.14 ± 4.51</td>
<td>-</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Note: pSS, primary Sjögren’s syndrome; ESSDAI, EULAR primary Sjögren’s syndrome disease activity. Unpaired t-test was used when comparing age, body weight and ESSDAI in pSS patients and healthy physical examiners. The sex ratio of pSS patients and healthy controls were analyzed using Fisher’s exact test.

Features in QC samples, i.e., the coefficient of variation, did not surpass 30% (**Figure 1C**). The similarity between samples was calculated using hierarchical clustering and the intra-group similarity between fecal and plasma samples was good (**Figure 1D**). Finally, the relative quantitative values of metabolites in feces and plasma were obtained by agglomerate hierarchical clustering (**Figure 1E**).

**Multivariate statistical analysis of LC-MS data**

Features in QC samples, i.e., the coefficient of variation, did not surpass 30% (**Figure 1C**). The similarity between samples was calculated using hierarchical clustering and the intra-group similarity between fecal and plasma samples was good (**Figure 1D**). Finally, the relative quantitative values of metabolites in feces and plasma were obtained by agglomerate hierarchical clustering (**Figure 1E**).

**Results**

The baseline characteristics of patients and healthy controls

Our cohort included 21 pSS patients and 18 healthy controls (HC) and their baseline characteristic was analyzed (**Table 1**). The difference in sex ratio, age and weight between pSS patients and HC was insignificant. In contrast, the ESSDAI of pSS patients was much higher than HC.

Quantification of metabolites in fecal and plasma samples by LC-MS

Fecal and plasma samples from pSS patients and HC were subjected to LC-MS. A typical base peak chromatogram showed the differences in the number, intensity and type of peaks in different samples, indicating that samples differed in both type and quantity of metabolites (**Figure 1A**). The dense distribution of QC samples on the PCA analysis plot indicated that the systematic error of the data was minimal and the data quality was reliable (**Figure 1B**). To discover biomarkers, poorly reproducible features in QC samples were removed to ensure that the relative standard deviation (RSD) of possible features in QC samples, i.e., the coefficient of variation, did not surpass 30% (**Figure 1C**). The similarity between samples was calculated using hierarchical clustering and the intra-group similarity between fecal and plasma samples was good (**Figure 1D**). Finally, the relative quantitative values of metabolites in feces and plasma were obtained by agglomerate hierarchical clustering (**Figure 1E**).

Identification of differential metabolites in fecal and plasma samples and pathway enrichment analysis

We screened for differential metabolites in fecal and plasma samples by p value ≤ 0.05 and VIP value ≥ 1 (**Supplementary Figures 1, 2**). There were 114 upregulated and 54 downregulated metabolites in the fecal samples and 92 upregulated and 125 downregulated metabolites in the plasma samples. Subsequently, a heatmap of the association between levels of differential metabolites within fecal or plasma samples was plotted to reflect the consistency of metabolite to metabolite (**Supplementary Figures 3, 4**).
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A. Base Peak Chromatogram

B. PCA score plot

C. PCA score plot

D. Heatmap of metabolite levels in S1, S2, S3, and HC groups

E. Heatmap of metabolite levels in different conditions
Metabolism-related biomarkers in pSS patients

Through the MetPA database, we detected that differential metabolites in feces were enriched in 92 pathways and differential metabolites in plasma were enriched in 120 pathways (Figure 3). Eight significantly enriched pathways in feces and 12 significant enriched pathways in plasma were screened according to Raw p (p-value of hypergeometric distribution test) < 0.05. Arginine biosynthesis, Linoleic acid metabolism, Tyrosine metabolism, Taurine and hypotaurine metabolism were common enriched pathways in both samples. This suggests that fecal metabolism is inextricably linked to plasma metabolism, pending further focus on these intersecting pathways.

Candidate diagnostic markers for pSS

To find specific differential metabolites that could be used as diagnostic markers, we first analyzed the intersecting metabolites of fecal and plasma samples enriched in the Arginine biosynthesis, Linoleic acid metabolism, Tyrosine metabolism, Taurine and hypotaurine metabolism pathways (Figure 4A). C00327 (Citrulline), C00437 (N-Acetylornithine), C14766 (9-OxoODE), C14829 (12,13-DHOME), C00483 (Tyramine), C00628 (Gentisic acid), C05123 (2-Hydroxyethanesulfonate) were found in the intersection. Citrulline, 9,10-12,13-Diepoxyoctadecanoate, Tyramine and Vanillylmandelic acid content was significantly decreased in pSS fecal samples (Figure 4B), while N-Acetylornithine, 9-OxoODE, 12,13-DHOME, Gentisic acid, Normetanephrine, L-Cysteine, Acetylphosphate showed opposite trends in pSS fecal and plasma samples. While 9,10-12,13-Diepoxyoctadecanoate, Tyramine, 9-OxoODE and 2-Hydroxyethanesulfonate showed the same trend in both samples of pSS patients. They were therefore recommended as candidate diagnostic markers to differentiate pSS patients from healthy populations.

Sensitivity analysis of pSS diagnostic markers

We observed that the AUC value for 9,10-12,13-Diepoxyoctadecanoate (Figure 5A), Tyramine (Figure 5B), 9-OxoODE (Figure 5C), and 2-Hydroxyethanesulfonate (Figure 5D) in fecal samples all ranged from 0.7 to 0.8 and the diagnostic effect had some accuracy. The AUC values of 9,10-12,13-Diepoxyoctadecanoate (Figure 5E) and Tyramine (Figure 5F) in plasma samples were above 0.9, indicating higher accuracy. The AUC values of 9-OxoODE (Figure 5G) ranged from 0.7 to 0.8 with average diagnostic efficacy. The AUC value of 2-Hydroxyethanesulfonate (Figure 5H) in plasma samples was greater than 0.9, with excellent sensitivity.

Discussion

pSS, a common systemic autoimmune disease, predominantly disturbs the lacrimal and salivary glands, contributing to dry eyes and mouth [21]. Recent advancement in understanding the pathogenesis of pSS enables the discovery of new biomarkers for the diagnosis and evaluation of disease activity [22]. Metabolomics has been used to examine changed metabolite profiles and to recognize metabolic signatures in diseases. Salivary biomarkers have been recently identified to discriminate pSS patients [23, 24]. Technological and methodological progresses have enabled the comprehensive characterization of human serum, urine, cerebrospinal fluid and saliva metabolomes, while the characterization of the human fecal metabolome lags behind other metabolomes regarding the availability of standardized methods and...
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Figure 2. Quality analysis of LC-MS data. A. Removal of Outliers and abnormal samples from fecal (left) and plasma (right) samples using PCA. B. PLS-DA of variability between fecal (left) and plasma (right) samples. C. PLS-DA of fecal (left) and plasma (right) samples for overfitting. D. OPLS-DA of the differences between groups. LC-MS, liquid chromatography-mass spectrometry; PLS-DA, partial least squares-discriminant analysis.
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freely available resources [25]. Systemic lupus erythematosus-altered fecal metabolites have been reported to be closely correlated with the indicators of this autoimmune disease [26, 27]. In this investigation, we displayed the possible ability of LC-MS-based metabolomics approach for discriminating pSS patients from HC on the basis of their fecal and plasma metabolite profiles. Four differential metabolic signatures, including 9,10-12,13-Diepoxyoctadecanoate, Tyramine, 9-OxoODE and 2-Hydroxyethanesulfonate, were identified in fecal and plasma samples of pSS patients.

LC-MS has been widely used to explore serum and urinary metabolomics in a vast network of diseases [20, 28]. In the present study, we subjected differential metabolic signatures derived from LC-MS to KEGG enrichment analysis to screen signaling pathways enriched by those metabolic signatures. Eight and twelve enriched pathways were filtered respectively from the fecal and plasma samples of pSS patients. Among them, arginine biosynthesis, linoleic acid metabolism, tyrosine metabolism, and taurine and hypotaurine metabolism were the common pathways. According to Łuczak et al, plasma L-arginine levels were much higher in pSS patients (n = 46, 29.07 ± 6.7 vs. 25.4 ± 5.23 µmol/L, P = 0.01) relative to controls (n = 30) [29]. Also, Castrejón-Morales et al. found a negative correlation between α-linoleic acid and the ESSDAI [30]. The reduced abundance of glycine, tyrosine, uric acid and fucose in salivary metabolites contributed to the loss of diversity in the pSS patients [31]. Taurine and hypotaurine metabolism have the potential to serve as predictive biomarkers for subcutaneous immunotherapy on seasonal allergic rhinitis by serum metabolomics [32]. This clinical evidence suggested that these metabolic pathways are closely linked to autoimmune diseases, particularly pSS, which supported our KEGG enrichment analysis results.

As far as the metabolic findings are concerned, in the multivariate model that characterized the profile of our patients, 9,10-12,13-Diepoxyoctadecanoate, Tyramine, 9-OxoODE and 2-Hydroxyethanesulfonate were identified as significant differential signatures showing the consistent content alteration in both samples. On the basis of HPLC-ESI-QTOF-MS methodology, the metabolic pathways altered in urinary and plasma samples of pSS patients were mainly associated with the metabolism of phospholipids, fatty acids, and amino acids, especially tryptophan, proline and phenylalanine [33]. 9,10-12,13-Diepoxyoctadecanoate and 9-OxoODE belong to linoleic acid metabolism. Lipid peroxidation was the main mechanism of ShengmaiSan intervention in Alzheimer’s disease, including suppression of the production of linoleic acid hydroperoxides, such as 9-OxoODE [34]. We also identified the enrichment of 9-OxoODE in the fecal and plasma samples of pSS patients. Tyramine refers to a biogenic trace amine that is produced through the decarboxylation of the amino acid tyrosine and it can control many physiological mechanisms at pico- to nanomolar concentrations, demonstrating neuro-modulatory properties and cardiovascular and immunological properties [35]. 2-Hydroxyethanesulfonate, on the other hand, belongs to taurine and hypotaurine metabolism. Taurine is a sulphur amino acid present at high concentrations in response to oxidative stress and it plays an antioxidant property in the immune system, protecting cells against oxidative stress [36]. It has been suggested by Castro et al. that Mucin 1 is overexpressed and accumulated in the endoplasmic reticulum of labial salivary gland from patients with SS, whereas tauoursodeoxycholic acid showed anti-inflammatory properties by decreasing Mucin 1 accumulation [37]. Furthermore, the ROC curves verified the sensitivities of these metabolites in predicting pSS, with more pronounced prediction effects in the plasma.
In conclusion, we observed that pSS is associated with an altered fecal and plasma metabolite profile when compared to healthy controls. The observations derived from this study may have implications in the pathogenesis or the diagnosis of the disease. Nevertheless, a potential pitfall of this work may be the relatively small size of our cohort. Larger studies are warranted to substantiate these results and to better highlight their functional implication.
Figure 5. The sensitivity of pSS diagnostic markers. ROC curve analysis of 9,10-12,13-Diepoxyoctadecanoate (A), Tyramine (B), 9-OxoODE (C), and 2-Hydroxyethanesulfonate (D) in fecal samples and 9,10-12,13-Diepoxyoctadecanoate (E), Tyramine (F), 9-OxoODE (G), 2-Hydroxyethanesulfonate (H) in plasma samples. pSS, primary Sjögren’s syndrome; ROC, relative operating characteristic.
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Disclosure of conflict of interest

None.

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Supplementary Figure 1. Differential metabolites in the fecal samples.
Supplementary Figure 2. Differential metabolites in the plasma samples.
Supplementary Figure 3. Correlation between levels of differential metabolites in the fecal samples.
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Supplementary Figure 4. Correlation between levels of differential metabolites in the plasma samples.