

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. Twelve metabolites were enriched in the above four pathways, while only 9,10-12,13-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 inter-

twine 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

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 ($\pm 1\%$) 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- μ m membrane for 10 min, and the filtrate was added to the assay bottle for LC-MS detection. Each sample to be tested (20 μ L) was mixed into quality control (QC) samples.

Plasma from patients and healthy controls was placed in a -80°C freezer and was later thawed on ice for experimentation. Each sample (100 μ L) was transferred into a centrifuge tube (2 mL), and supplemented with 400 μ L 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/>), Lipid-Maps (<http://www.hmdb.ca>), and mzcloud (<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

Table 1. Baseline characteristics of pSS patients and healthy controls

Characteristics		pSS patients (n = 21)	Healthy control (n = 18)	p value
Sex	Female	19	15	0.647
	male	2	3	
Age (year)		56.42 ± 12.41	54.5 ± 8.7	0.599
Body weight (kg)		62.71 ± 10.63	63.29 ± 11.2	0.869
ESSDAI		10.14 ± 4.51	-	< 0.001

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.

were displayed as mean ± SD. Unpaired t-test was applied when comparing age, body weight and ESSDAI in pSS patients and healthy physical examiners. The sex ratio of pSS patients and healthy physical examinees was analyzed using Fisher's exact test. AUC was applied to determine the significance threshold for the sensitivity of pSS biomarkers. $P < 0.05$ was considered statistically significant.

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**).

Multivariate statistical analysis of LC-MS data

New characteristic variables were generated using linear combination of metabolite variables based on certain weights through PCA and the groups of data were categorized by the main new variables (principal components) to remove poorly reproducible samples (outlier samples) and abnormal samples (samples outside the confidence interval - Hotelling T2 ellipse) (**Figure 2A**). We observed a high degree of dispersion between fecal and plasma samples and a low degree of dispersion within fecal and plasma samples. When the samples were specified and grouped, we observed the variability between the different groups of samples more visually by PLS-DA analysis (**Figure 2B**) and the results were not overfitting (**Figure 2C**). Finally, OPLS-DA was used to maximize the differences between groups (**Figure 2D**). The fecal and plasma-like variable importance for the projection (VIP) values calculated on the basis of the PLS-DA model was used for subsequent differential metabolite analysis.

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**).

Metabolism-related biomarkers in pSS patients

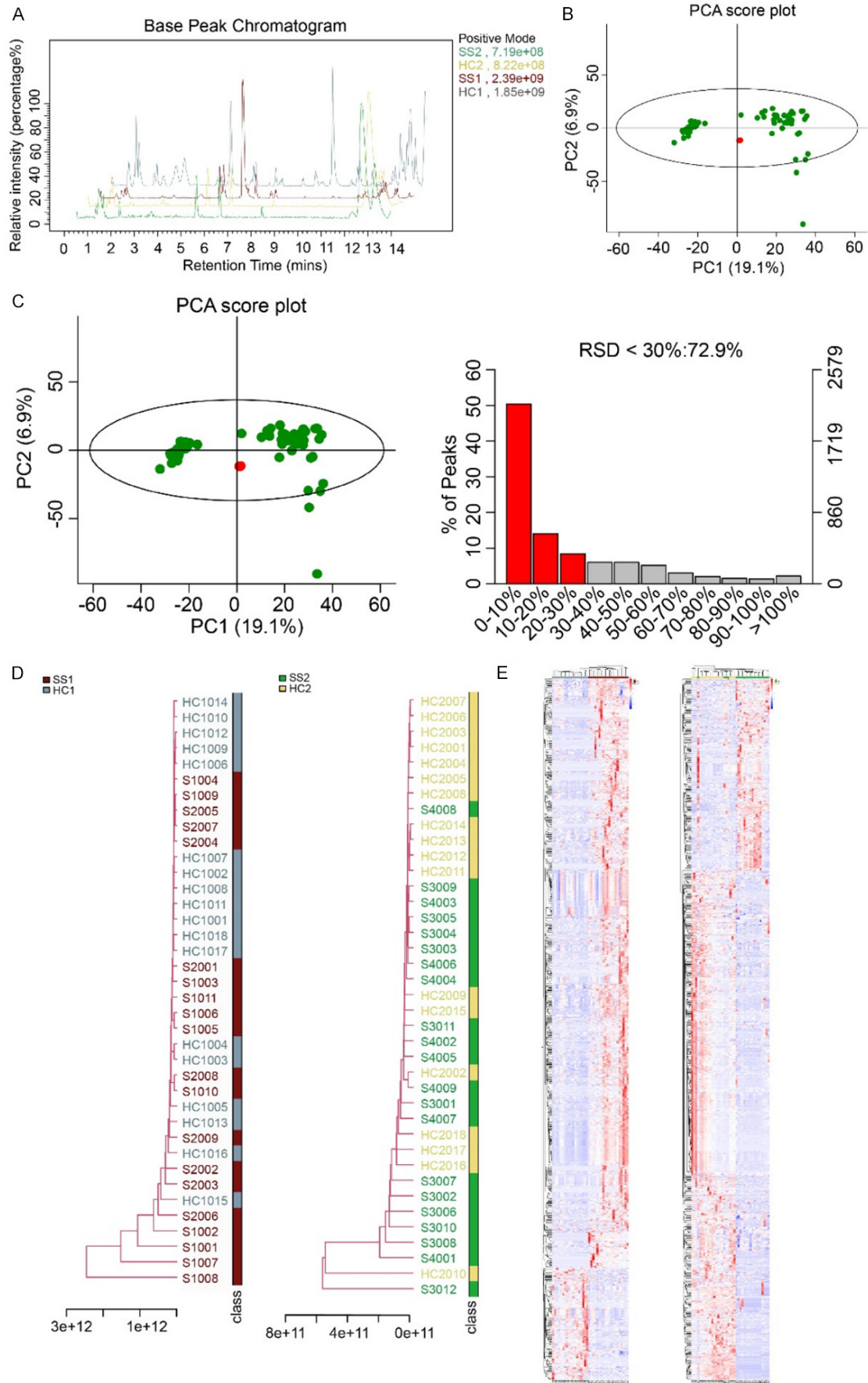


Figure 1. Metabolite levels in fecal and plasma samples from pSS patients and HC. A. Base peak chromatogram showing differences in metabolite abundance in different samples. B. PCA plot of QC samples. C. Coefficient of variation of the samples. D. Similarity between fecal (left) and plasma (right) samples. E. Relative quantitative values of metabolites in feces (left) and plasma (right). pSS, primary Sjögren's syndrome; HC, healthy control; PCA, principal component analysis; QC, quality control.

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), C14836 (9,10-12,13-Diepoxyoctadecanoate), C00483 (Tyramine), C00628 (Gentisic acid), C05584 (Vanillylmandelic acid), C05589 (Nor-metanephrene), C00097 (L-Cysteine), C00227 (Acetylphosphate), 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, Normetanephrene, L-Cysteine, Acetylphosphate and 2-Hydroxyethanesulfonate were significantly increased (**Figure 4C**). Significantly lower levels of N-Acetylornithine, 12,13-DHOME, 9,10-12,13-Diepoxyoctadecanoate, Tyramine, Gentisic acid, Normetanephrene, L-Cysteine, and Acetylphosphate were observed in pSS plasma samples (**Figure 4D**). By contrast, the enhanced levels of Citrulline, 9-OxoODE, Vanillylmandelic acid and 2-Hydroxyethanesulfonate were detected in the plasma samples of pSS patients (**Figure 4E**).

In conclusion, the levels of Citrulline, Vanillylmandelic acid, N-Acetylornithine, 12,13-DHOME, Gentisic acid, Normetanephrene, 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

Metabolism-related biomarkers in pSS patients

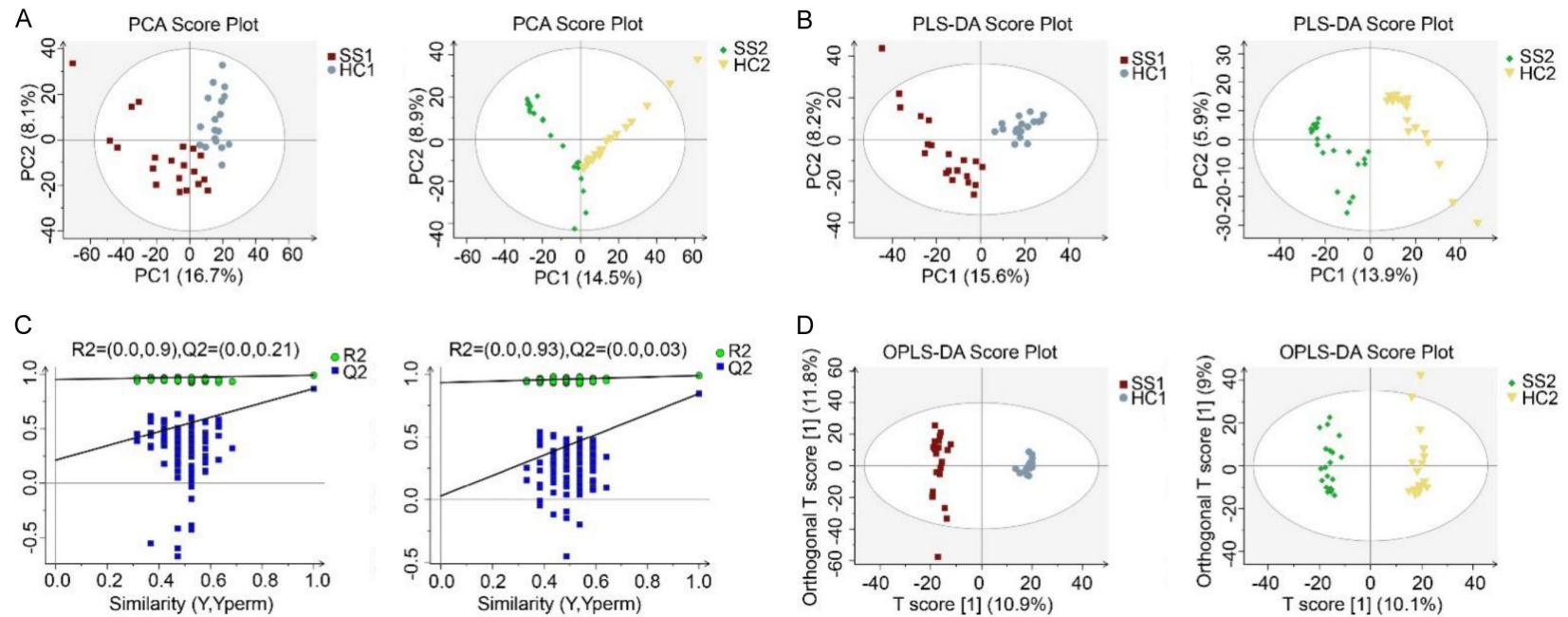


Figure 2. Quality analysis of LC-MS data. A. Removal of Outliers and abnormal samples from fecal (left) and plasma (right) samples using PCA. B. PLS-DA of variability between fecal (left) and plasma (right) samples. C. PLS-DA of fecal (left) and plasma (right) samples for overfitting. D. OPLS-DA of the differences between groups. LC-MS, liquid chromatography-mass spectrometry; PLS-DA, partial least squares-discriminant analysis.

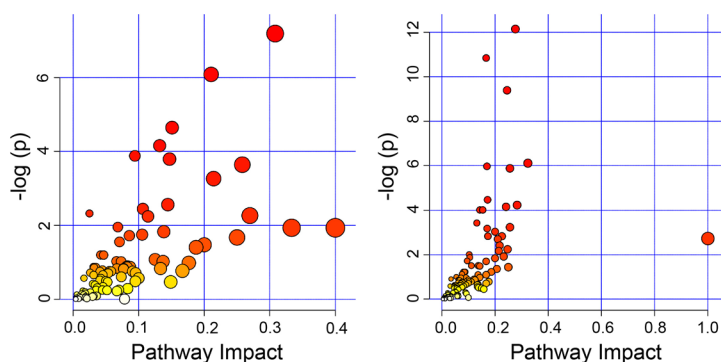


Figure 3. Pathways enriched by differential metabolites in fecal (left) and plasma samples (right).

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 $\mu\text{mol/L}$, $P = 0.01$) 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 rhini-

tis 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 signa-

tures 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 concentration 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 tauroursodeoxycholic 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.

Metabolism-related biomarkers in pSS patients

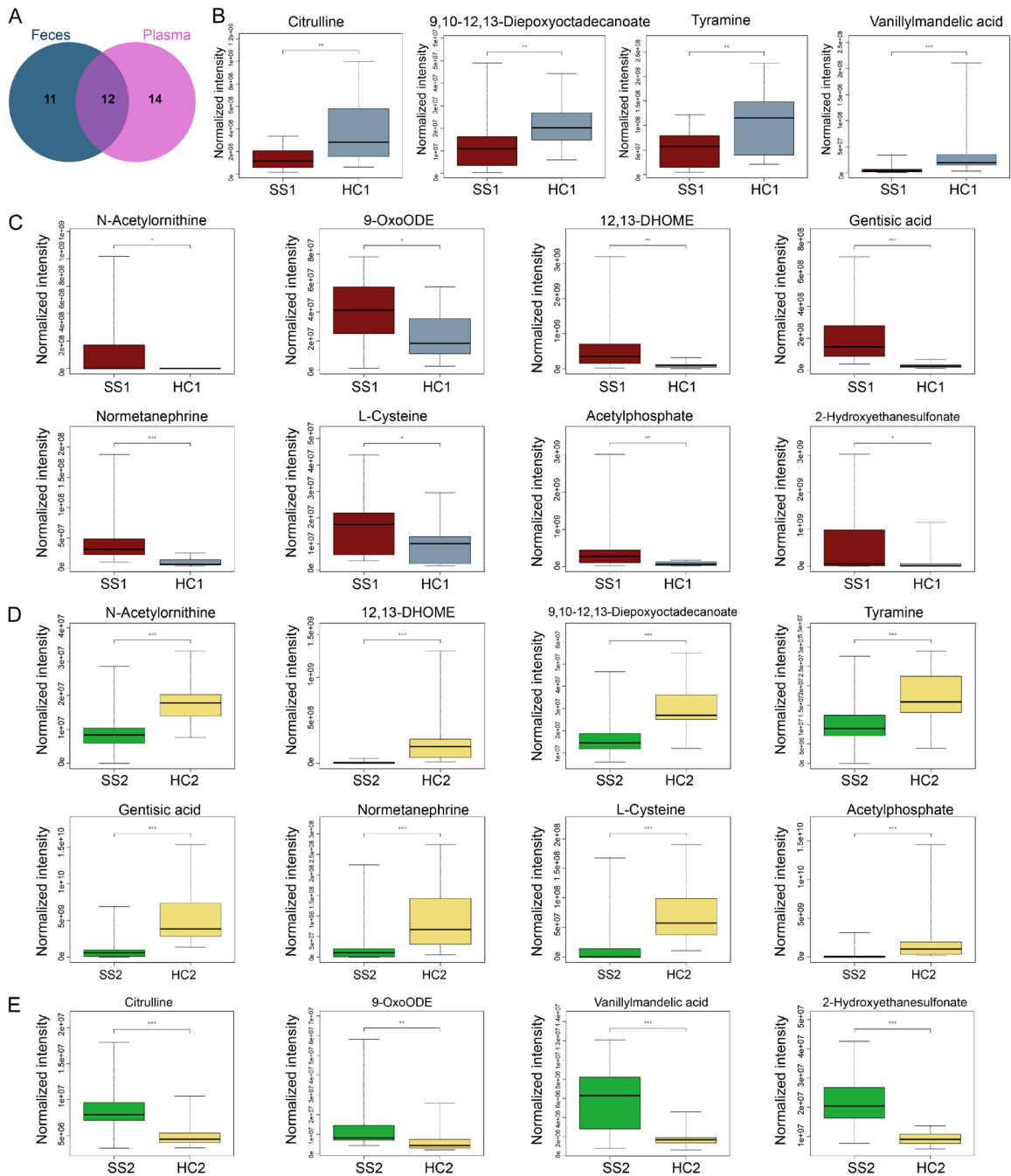


Figure 4. Screening of candidate diagnostic markers for pSS. A. Intersected metabolites in fecal and plasma samples. B. Metabolites with reduced abundance in pSS fecal samples. C. Metabolites with enhanced abundance in pSS fecal samples. D. Metabolites with reduced abundance in pSS plasma samples. E. Metabolites with enhanced abundance in pSS plasma samples. pSS, primary Sjögren's syndrome.

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.

Metabolism-related biomarkers in pSS patients

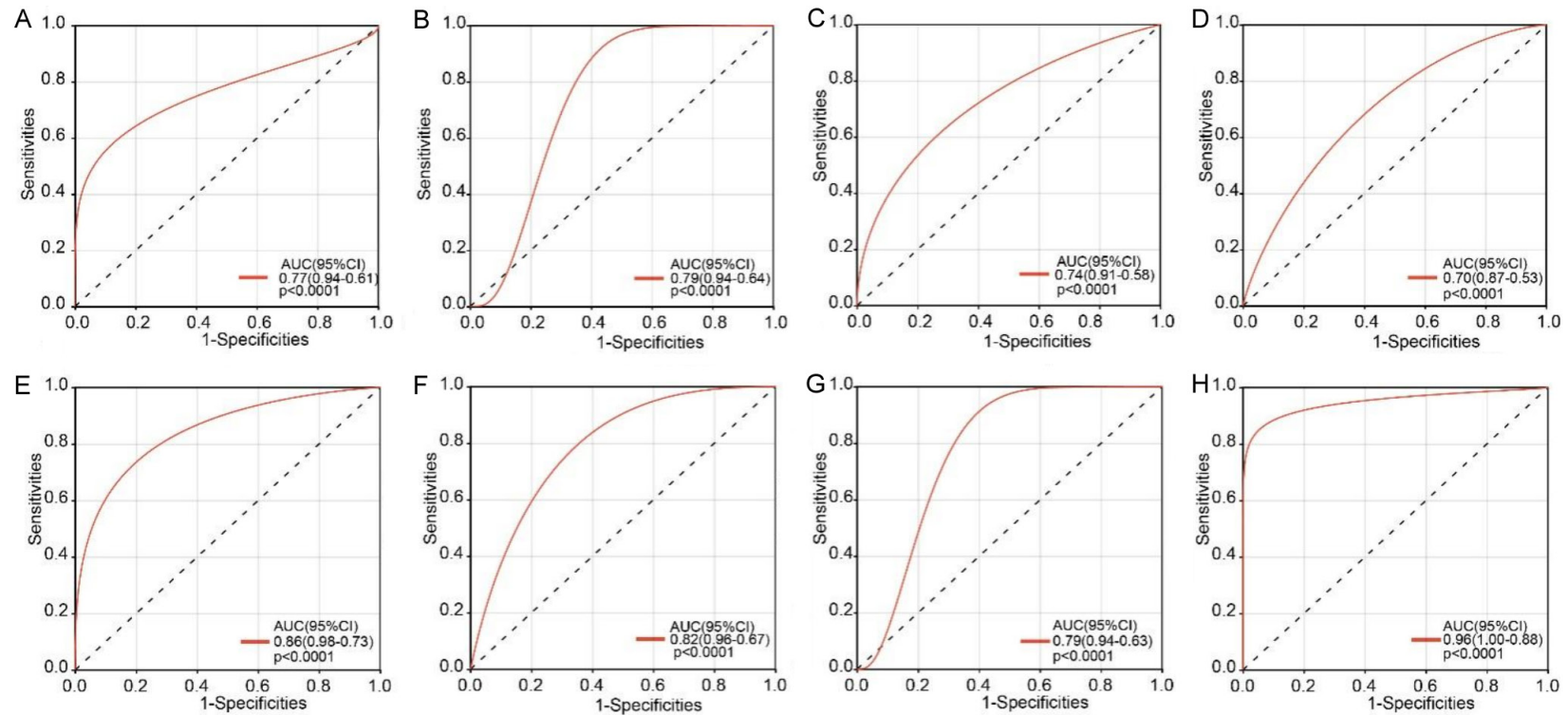


Figure 5. The sensitivity of pSS diagnostic markers. ROC curve analysis of 9,10-12,13-Diepoxyoctadecanoate (A), Tyramine (B), 9-OxoODE (C), and 2-Hydroxyethanesulfonate (D) in fecal samples and 9,10-12,13-Diepoxyoctadecanoate (E), Tyramine (F), 9-OxoODE (G), 2-Hydroxyethanesulfonate (H) in plasma samples. pSS, primary Sjögren's syndrome; ROC, relative operating characteristic.

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Disclosure of conflict of interest

None.

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References

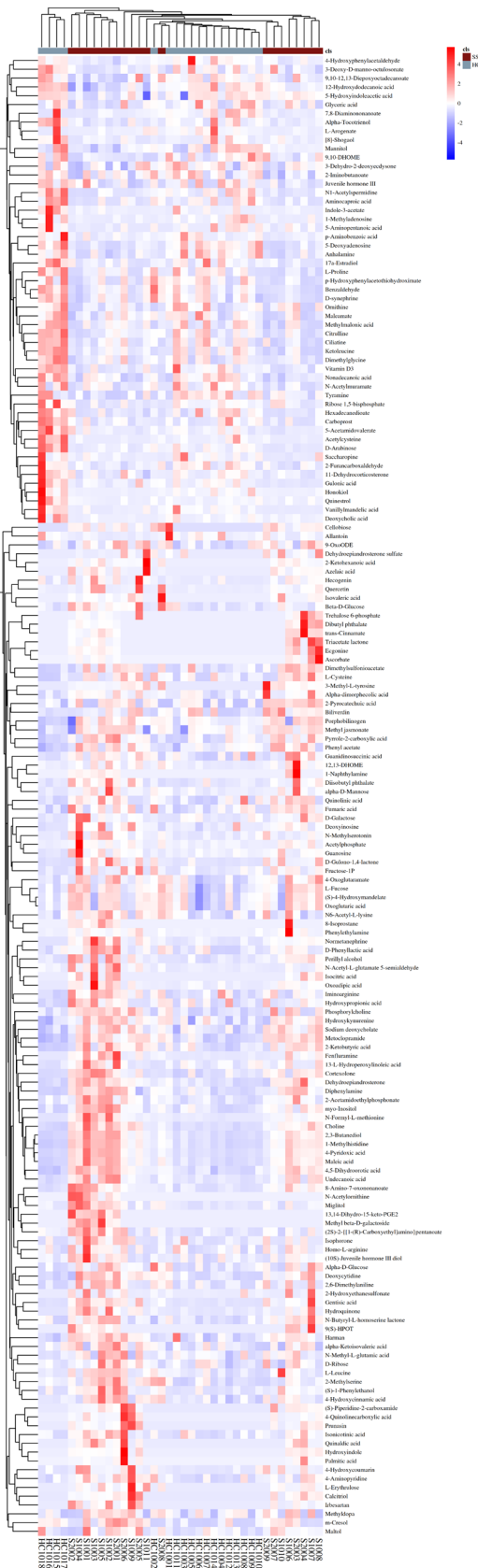
- [1] Pontarini E, Lucchesi D and Bombardieri M. Current views on the pathogenesis of Sjogren's syndrome. *Curr Opin Rheumatol* 2018; 30: 215-221.
- [2] Ramos-Casals M, Brito-Zeron P, Siso-Almirall A and Bosch X. Primary Sjogren syndrome. *BMJ* 2012; 344: e3821.
- [3] Brito-Zeron P, Baldini C, Bootsma H, Bowman SJ, Jonsson R, Mariette X, Sivils K, Theander E, Tzioufas A and Ramos-Casals M. Sjogren syndrome. *Nat Rev Dis Primers* 2016; 2: 16047.
- [4] Mariette X and Criswell LA. Primary Sjogren's syndrome. *N Engl J Med* 2018; 378: 931-939.
- [5] Ramponi G, Folci M, Badalamenti S, Angelini C and Brunetta E. Biomarkers and diagnostic testing for renal disease in Sjogren's syndrome. *Front Immunol* 2020; 11: 562101.
- [6] Agrawi LA, Galtung HK, Vestad B, Ovstebo R, Thiede B, Rusthen S, Young A, Guerreiro EM, Utheim TP, Chen X, Utheim OA, Palm O and Jensen JL. Identification of potential saliva and tear biomarkers in primary Sjogren's syndrome, utilising the extraction of extracellular vesicles and proteomics analysis. *Arthritis Res Ther* 2017; 19: 14.
- [7] Vitali C, Minniti A, Pignataro F, Maglione W and Del Papa N. Management of Sjogren's syndrome: present issues and future perspectives. *Front Med (Lausanne)* 2021; 8: 676885.
- [8] Chu X, Jaeger M, Beumer J, Bakker OB, Aguirre-Gamboa R, Oosting M, Smeekens SP, Moorlag S, Mourits VP, Koeken V, de Bree C, Jansen T, Mathews IT, Dao K, Najhawan M, Watrous JD, Joosten I, Sharma S, Koenen H, Withoff S, Jonkers IH, Netea-Maier RT, Xavier RJ, Franke L, Xu CJ, Joosten LAB, Sanna S, Jain M, Kumar V, Clevers H, Wijmenga C, Netea MG and Li Y. Integration of metabolomics, genomics, and immune phenotypes reveals the causal roles of metabolites in disease. *Genome Biol* 2021; 22: 198.
- [9] Di Minno A, Gelzo M, Caterino M, Costanzo M, Ruoppolo M and Castaldo G. Challenges in metabolomics-based tests, biomarkers revealed by metabolomic analysis, and the promise of the application of metabolomics in precision medicine. *Int J Mol Sci* 2022; 23: 5213.
- [10] Vuckovic D. Current trends and challenges in sample preparation for global metabolomics using liquid chromatography-mass spectrometry. *Anal Bioanal Chem* 2012; 403: 1523-1548.
- [11] Urbanski G, Assad S, Chabrun F, Chao de la Barca JM, Blanchet O, Simard G, Lenaers G, Prunier-Mirebeau D, Gohier P, Lavigne C and Reynier P. Tear metabolomics highlights new potential biomarkers for differentiating between Sjogren's syndrome and other causes of dry eye. *Ocul Surf* 2021; 22: 110-116.
- [12] Zoukhri D, Rawe I, Singh M, Brown A, Kublin CL, Dawson K, Haddon WF, White EL, Hanley KM, Tuse D, Malyj W and Papas A. Discovery of putative salivary biomarkers for Sjogren's syndrome using high resolution mass spectrometry and bioinformatics. *J Oral Sci* 2012; 54: 61-70.
- [13] Fernandez-Ochoa A, Brunius C, Borrás-Linares I, Quirantes-Pine R, Cadiz-Gurrea ML, Precisesads Clinical C, Alarcon Riquelme ME and Segura-Carretero A. Metabolic disturbances in urinary and plasma samples from seven different systemic autoimmune diseases detected by HPLC-ESI-QTOF-MS. *J Proteome Res* 2020; 19: 3220-3229.
- [14] Zhang Q, Yin X, Wang H, Wu X, Li X, Li Y, Zhang X, Fu C, Li H and Qiu Y. Fecal metabolomics and potential biomarkers for systemic lupus erythematosus. *Front Immunol* 2019; 10: 976.
- [15] Mandal R, Cano R, Davis CD, Hayashi D, Jackson SA, Jones CM, Lampe JW, Latulippe ME, Lin NJ, Lippa KA, Piotrowski P, Da Silva SM, Swanson KS and Wishart DS. Workshop report: toward the development of a human whole stool reference material for metabolomic and metagenomic gut microbiome measurements. *Metabolomics* 2020; 16: 119.
- [16] Mandl T, Marsal J, Olsson P, Ohlsson B and Andreasson K. Severe intestinal dysbiosis is prevalent in primary Sjogren's syndrome and is associated with systemic disease activity. *Arthritis Res Ther* 2017; 19: 237.
- [17] Shiboski CH, Shiboski SC, Seror R, Criswell LA, Labetoulle M, Lietman TM, Rasmussen A, Scofield H, Vitali C, Bowman SJ and Mariette X;

- International Sjogren's Syndrome Criteria Working Group. 2016 American college of rheumatology/european league against rheumatism classification criteria for primary Sjogren's syndrome: a consensus and data-driven methodology involving three international patient cohorts. *Ann Rheum Dis* 2017; 76: 9-16.
- [18] Seror R, Bootsma H, Saraux A, Bowman SJ, Theander E, Brun JG, Baron G, Le Guern V, Devauchelle-Pensec V, Ramos-Casals M, Valim V, Dorner T, Tzioufas A, Gottenberg JE, Solans Laque R, Mandl T, Hachulla E, Sivils KL, Ng WF, Fauchais AL, Bombardieri S, Priori R, Bartoloni E, Goeb V, Praprotnik S, Sumida T, Nishiyama S, Caporali R, Kruize AA, Vollenweider C, Ravaud P, Meiners P, Brito-Zeron P, Vitali C and Mariette X; EULAR Sjögren's Task Force. Defining disease activity states and clinically meaningful improvement in primary Sjogren's syndrome with EULAR primary Sjogren's syndrome disease activity (ESSDAI) and patient-reported indexes (ESSPRI). *Ann Rheum Dis* 2016; 75: 382-389.
- [19] Navazesh M and Kumar SK; University of Southern California School of Dentistry. Measuring salivary flow: challenges and opportunities. *J Am Dent Assoc* 2008; 139 Suppl: 35S-40S.
- [20] Thevenot EA, Roux A, Xu Y, Ezan E and Junot C. Analysis of the human adult urinary metabolome variations with age, body mass index, and gender by implementing a comprehensive workflow for univariate and opls statistical analyses. *J Proteome Res* 2015; 14: 3322-3335.
- [21] Du W, Han M, Zhu X, Xiao F, Huang E, Che N, Tang X, Zou H, Jiang Q and Lu L. The multiple roles of B cells in the pathogenesis of Sjogren's syndrome. *Front Immunol* 2021; 12: 684999.
- [22] Chen W, Cao H, Lin J, Olsen N and Zheng SG. Biomarkers for primary Sjogren's syndrome. *Genomics Proteomics Bioinformatics* 2015; 13: 219-223.
- [23] Herrala M, Turunen S, Hanhineva K, Lehtonen M, Mikkonen JJW, Seitsalo H, Lappalainen R, Tjaderhane L, Niemela RK, Salo T, Myllymaa S, Kullaa AM and Karkkainen O. Low-dose doxycycline treatment normalizes levels of some salivary metabolites associated with oral microbiota in patients with primary Sjogren's syndrome. *Metabolites* 2021; 11: 595.
- [24] Jung JY, Kim JW, Kim HA and Suh CH. Salivary biomarkers in patients with Sjogren's syndrome-a systematic review. *Int J Mol Sci* 2021; 22: 12903.
- [25] Karu N, Deng L, Slae M, Guo AC, Sajed T, Huynh H, Wine E and Wishart DS. A review on human fecal metabolomics: methods, applications and the human fecal metabolome database. *Anal Chim Acta* 2018; 1030: 1-24.
- [26] Wen M, Liu T, Zhao M, Dang X, Feng S, Ding X, Xu Z, Huang X, Lin Q, Xiang W, Li X, He X and He Q. Correlation analysis between gut microbiota and metabolites in children with systemic lupus erythematosus. *J Immunol Res* 2021; 2021: 5579608.
- [27] Yan R, Jiang H, Gu S, Feng N, Zhang N, Lv L and Liu F. Fecal metabolites were altered, identified as biomarkers and correlated with disease activity in patients with systemic lupus erythematosus in a GC-MS-based metabolomics study. *Front Immunol* 2020; 11: 2138.
- [28] Li J, Che N, Xu L, Zhang Q, Wang Q, Tan W and Zhang M. LC-MS-based serum metabolomics reveals a distinctive signature in patients with rheumatoid arthritis. *Clin Rheumatol* 2018; 37: 1493-1502.
- [29] Luczak A, Malecki R, Kulus M, Madej M, Szahidewicz-Krupska E and Doroszko A. Cardiovascular risk and endothelial dysfunction in primary Sjogren syndrome is related to the disease activity. *Nutrients* 2021; 13: 2072.
- [30] Castrejon-Morales CY, Granados-Portillo O, Cruz-Bautista I, Ruiz-Quintero N, Manjarrez I, Lima G, Hernandez-Ramirez DF, Astudillo-Angel M, Llorente L and Hernandez-Molina G. Omega-3 and omega-6 fatty acids in primary Sjogren's syndrome: clinical meaning and association with inflammation. *Clin Exp Rheumatol* 2020; 38 Suppl 126: 34-39.
- [31] Kageyama G, Saegusa J, Irino Y, Tanaka S, Tsuda K, Takahashi S, Sendo S and Morinobu A. Metabolomics analysis of saliva from patients with primary Sjogren's syndrome. *Clin Exp Immunol* 2015; 182: 149-153.
- [32] Yu RL, Pan C, Ma TT, Wang XY, Shi HY, Zhuang Y, Yan WJ, Liu JG, Cao MD, Sun JL, Wang DY, Yin JS, Wei JF and Wang XY. Prediction of clinical efficacy of subcutaneous immunotherapy for *Artemisia sieversiana* pollen allergic rhinitis by serum metabolomics. *J Formos Med Assoc* 2022; [Epub ahead of print].
- [33] Fernandez-Ochoa A, Borrás-Linares I, Quirantes-Pine R, Alarcon-Riquelme ME, Beretta L and Segura-Carretero A; Precisesads Clinical Consortium. Discovering new metabolite alterations in primary Sjogren's syndrome in urinary and plasma samples using an HPLC-ESI-QTOF-MS methodology. *J Pharm Biomed Anal* 2020; 179: 112999.
- [34] Lu S, Han Y, Chu H, Kong L, Zhang A, Yan G, Sun H, Wang P and Wang X. Characterizing serum metabolic alterations of Alzheimer's disease and intervention of Shengmai-San by ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry. *Food Funct* 2017; 8: 1660-1671.

Metabolism-related biomarkers in pSS patients

- [35] Andersen G, Marcinek P, Sulzinger N, Schieberle P and Krautwurst D. Food sources and biomolecular targets of tyramine. *Nutr Rev* 2019; 77: 107-115.
- [36] Gambino CM, Di Bona D, Aiello A, Carru C, Duro G, Guggino G, Ferrante A, Zinellu A, Caruso C, Candore G and Accardi G. HLA-C1 ligands are associated with increased susceptibility to systemic lupus erythematosus. *Hum Immunol* 2018; 79: 172-177.
- [37] Castro I, Albornoz N, Aguilera S, Barrera MJ, Gonzalez S, Nunez M, Carvajal P, Jara D, Lagos C, Molina C, Urzua U, Hermoso MA and Gonzalez MJ. Aberrant MUC1 accumulation in salivary glands of Sjogren's syndrome patients is reversed by TUDCA in vitro. *Rheumatology (Oxford)* 2020; 59: 742-753.

Metabolism-related biomarkers in pSS patients



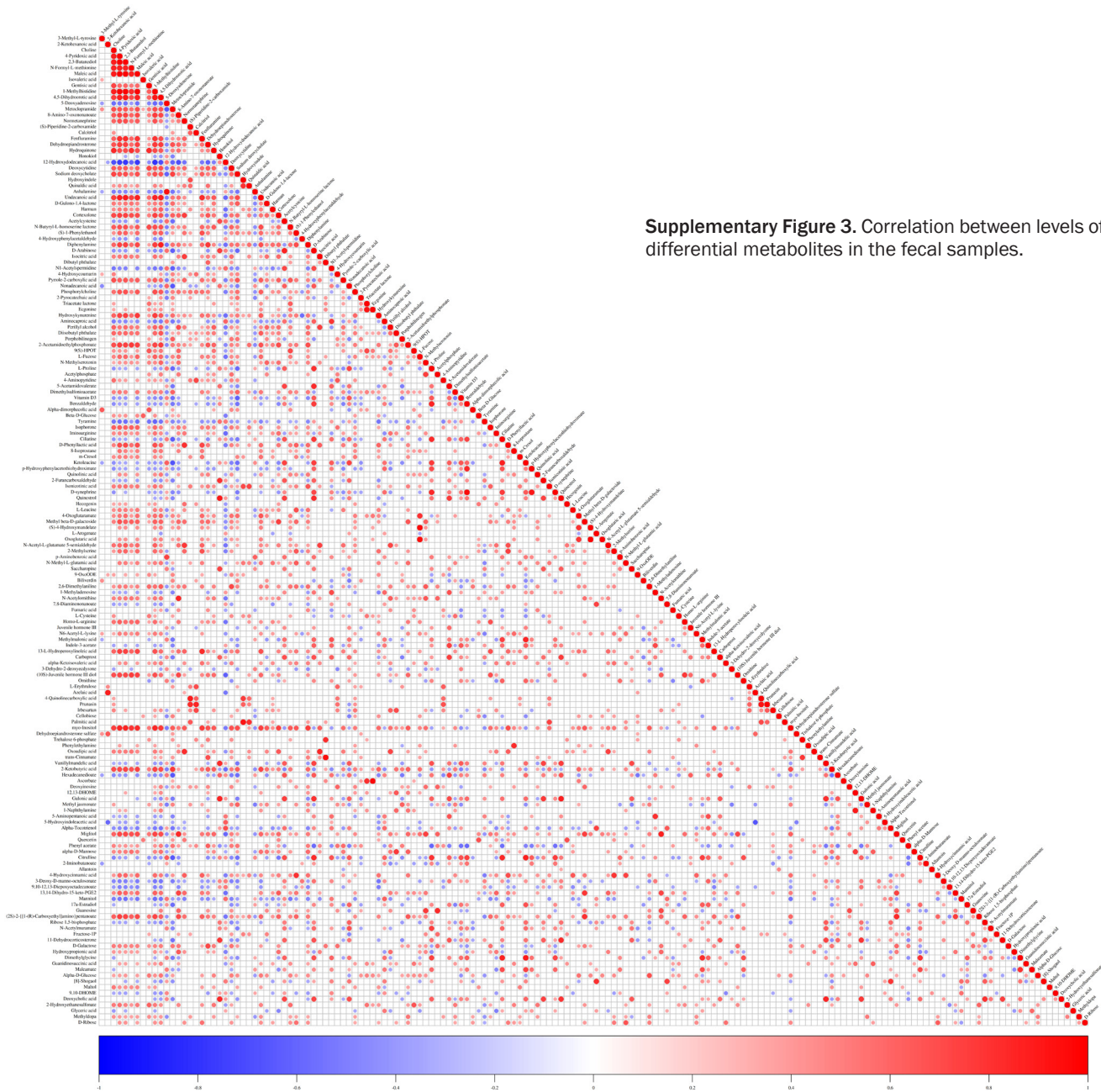
Supplementary Figure 1. Differential metabolites in the fecal samples.

Metabolism-related biomarkers in pSS patients



Supplementary Figure 2. Differential metabolites in the plasma samples.

Metabolism-related biomarkers in pSS patients



Metabolism-related biomarkers in pSS patients

