Untargeted serum metabolomics and potential biomarkers for Sjögren's syndrome

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ABSTRACT

Objective. *At present, the pathogenesis of Sjögren's syndrome (SS) remains unclear. This research aimed to identify differential metabolites that contribute to SS diagnosis and discover the disturbed metabolic pathways.*

Methods. *Recent advances in mass spectrometry have allowed the identification of hundreds of unique metabolic signatures and the exploration of altered metabolite profiles in disease. In this study, 505 candidates including healthy controls (HCs) and SS patients were recruited and the serum samples were collected. A non-targeted gas chromatography-mass spectrometry (GC-MS) serum metabolomics method was used to explore the changes in serum metabolites.*

Results. *We found SS patients and HCs can be distinguished by 21 significant metabolites. The levels of alanine, tryptophan, glycolic acid, pelargonic acid, cis-1-2-dihydro-1-2-naphthalenediol, diglycerol, capric acid, turanose, behenic acid, dehydroabietic acid, stearic acid, linoleic acid, heptadecanoic acid, valine, and lactic acid were increased in serum samples from SS patients, whereas levels of catechol, anabasine, 3-6-anhydro-D-galactose, beta-gentiobiose, 2-ketoisocaproic acid and ethanolamine were decreased. The significantly changed pathways included the following: Linoleic acid metabolism; unsaturated fatty acid biosynthesis; aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis; glycerolipid metabolism; selenocompound metabolism; galactose metabolism; alanine, aspartate and glutamate metabolism; glyoxylate and dicarboxylate metabolism; glycerophospholipid metabolism; and valine, leucine and isoleucine degradation.*

Conclusion. *These findings enhance the informative capacity of biochemical analyses through the identification of serum biomarkers and the analysis of metabolic pathways and contribute to an improved understanding of the pathogenesis of SS.*

Introduction

Sjögren's syndrome (SS) is an autoimmune disease characterised by lymphocytic invasion of the salivary and lacrimal glands, with B-cell hyperactivity. The main clinical manifestations of SS are dry eye and oral dryness (1, 2). SS includes primary (pSS) and secondary SS, secondary is related to systemic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus or systemic sclerosis (3). SS affects men and women at a ratio of 1:9 (4), and approximately 1-3% of the general population develops SS (5). Although the disease can occur at any age, it usually develops in menopausal women in the fourth and fifth decades of life (6, 7).

Advances in the understanding of SS pathophysiology have been achieved with omics analyses. For example, Fernández-Ochoa *et al*. (8) identified significant changes in the tryptophan, proline, and phenylalanine levels related to the phospholipid, fatty acid, and amino acid metabolic pathways in patients with SS. Kageyama *et al*. (9) found that the diversity of salivary metabolites are lower in patients with pSS than in healthy controls (HCs) and that the presence of major salivary glanditis influences the metabolite profile in patients with pSS. Novel data obtained in recent years have supported initial studies investigating primary biomarkers of SS in patients' tears and saliva (10). According to Versura *et al*. (11), the protein concentrations of LACTO and LIPOC-1 in tears have shown promising diagnostic performance in traditional ocular clinical trials, and these factors can be considered potential bio-

markers of SS. Furthermore, Jazzar *et al*. (12) found significant differences in the salivary levels of S100A8/A9 in a lymphoma risk-based subgroup of patients with SS.

In recent years, many reports regarding the application of metabolomics for the investigation of the pathologic mechanisms of systemic autoimmune diseases (SADs) have been published (13, 14). The appropriate treatment for patients with SS remains controversial due to the unavailability of unique and effective treatments (15). Analysis of the changes in serum metabolites by mass spectrometry can discover new molecules that might play a key role in SS, which would contribute to an improved understanding of the pathogenesis of the disease and improve its clinical diagnosis.

In this study, the serum metabolite profiles of patients with SS patients and HCs were studied using a non-targeted GC-MS serum metabolomics method combined with multivariate and univariate statistical analyses to explore the metabolomic profiles of patients with SS and HCs.

Methods

Ethics approval and consent to participate

The investigation scheme was approved by the ethics committee of the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (2018NL-122-02). Signed consent forms were obtained from all the study subjects and treated patients who provided samples after they reviewed a written plan of the entire study. All projects were in compliance with the ethical standards of the Institutional Review Board and the Ethics Committee of the First Affiliated Hospital of Nanjing University of Traditional Chinese.

Study population and characteristics

Between November 2018 and October 2019, 209 patients who met the revised American-European Consensus Group (AECG) classification criteria for SS were included (16). All patients were enrolled at the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. The patients' clinical information, including sex, age, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), immunoglobulin G (IgG), immunoglobulin A (IgA), anti-SSA/Ro autoantibodies (SSA), anti-SSB/La autoantibodies (SSB) and anti-nuclear antibody (ANA) values, is shown in Supplementary Table S1. Ninety patients (male/female: 1/10) and 153 controls (male/female: 1/10) were allocated to the discovery set, and 119 patients (male/female: 1/10) and 143 controls (male/female: 1/10) were allocated to the validation set. All serum samples were stored at -80 °C until analysis.

Materials

Liquid chromatography-grade acetonitrile (ACN), methanol (MeOH) and methyl tert-butyl ether (MTBE) were purchased from Merck (Darmstadt, Germany), and isopropyl alcohol, formic acid and ammonium formate were purchased from ROE (DE, USA). Ultrapure water was obtained with a Milli-Q system (Millipore, Billerica, MA, USA). N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), methoxyamine hydrochloride, pyridine and 1,2-$^{13}C_2$-myristic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation and analysis

The plasma samples were first thawed on ice. Briefly, 225 µl of ice-cold methanol was added to 40 µl serum; and the mixture was vortexed for 10 s. Subsequently 750 µl of cold MTBE was added, and the mixture was vortexed for 10 seconds and incubated at 4°C for 10 min in a Thermo Mixer C (Eppendorf, Hamburg, Germany). After the addition of 188 µl of ultrapure water, the mixture was vortexed for 20 seconds and centrifuged for 2 min (14,000 rcf, 4°C). The supernatant was then combined with 750 µl of methanol: isopropanol (1:1), and the mixture was shaken for 10 min in an orbital mixer and centrifuged for 2 min (14,000 rcf, 4°C). Subsequently, 475 µl of the supernatant was dried in a SpeedVac sample concentrator at 45°C for 2 h. The dried aliquots were combined with 1 µl of 1,2-$^{13}C_2$-myristic acid (5 mg/ml) as an internal standard and 30 µl of methoxyamine hydrochloride in pyridine (10 mg/ml), and the mixture was then vortexed for 1 min and shaken at 30°C for 90 min in a shaker. Thirty microliters of BSTFA containing 1% TMCS was added to the sample, and the mixture was shaken at 37 °C for 30 min, transferred to a sample vial with a glass insert and analysed by GC-MS. The order in which the samples from the patients with SS and HCs were analysed was random, and quality control samples were injected once per batch of 10 samples.

Chromatographic separation was performed using a Trace 1310 gas chromatograph equipped with an AS 1310 autosampler. A full scan was performed with a TSQ 8000 triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA), as described previously by Xie *et al*. (17) For the analysis of each sample, a single injection of a standard n-alkane mixture (C8-C40) was used to detect and eliminate retention time shifts.

Data analysis

The raw data obtained from Xcalibur 2.2 software (Thermo Scientific) were converted to "abf" format with the ABF converter (http://www.reifycs.com/AbfConverter/index.html). MS-DIAL (18) with FiehnLib was employed for peak detection, alignment and identification. The parameter settings were as follows: peak detection; smoothing method, linear weighted moving average; smoothing level, 2 scan; average peak width, 20 scan; and minimum peak height, 1000 amplitude. The deconvolution parameters were as follows: sigma window value, 0.5; and EI spectra cut off, 10 amplitudes. The identification settings were as follows: use retention index; MSP file, FiehnLib database (19); RI tolerance, 3000; RT tolerance, 0.5; m/z tolerance, 0.5; EI similarity cut off, 70%; and identification score cut off, 70%. The spectra were matched against the mass spectra and retention index library FiehnLib.

Normalisation of the intensity of each ionic feature was achieved using MetaboAnalyst 3.0 (https://www.metaboanalyst.ca/). We reduced the resulting ma-

trix by replacing all the missing values with a small value. Partial least squares-discriminant analysis (PLS-DA) was performed using SIMCA software (version 13.0; Umetrics). The significantly altered metabolites were selected according to their variable importance for projection (VIP >1.0) values and Student's t-test (p<0.05). The Hochberg-Benjamini method was applied to obtain the false discovery rate (FDR), and an FDR <0.05 was considered significant. Binary logistic regression (BLR) and receiver operating characteristic (ROC) curve analyses of the differential metabolites were performed with SPSS (IBM SPSS Statistics 22.0) to develop a BLR model for patients with SS and evaluate its clinical diagnosis capacity.

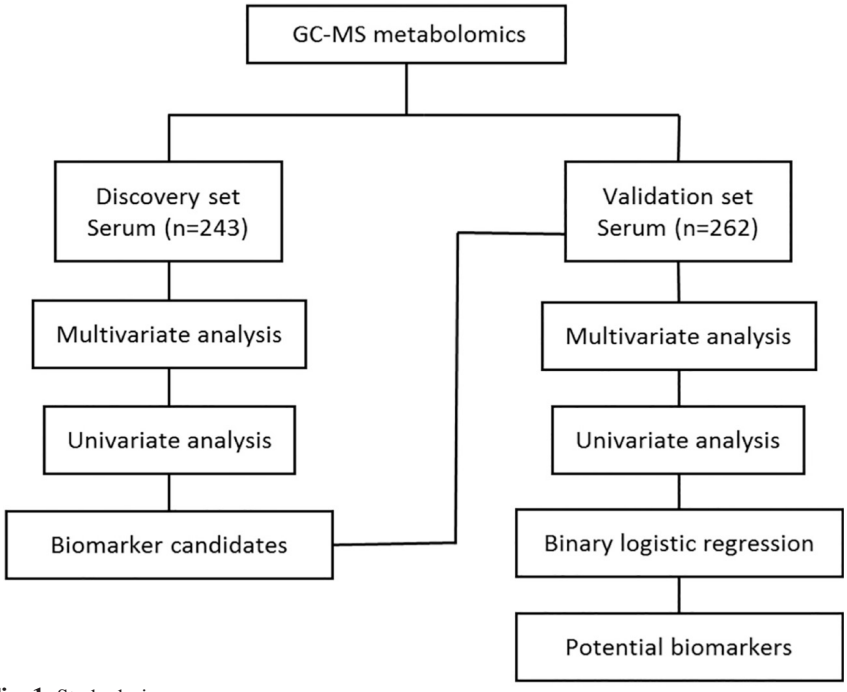

Fig. 1. Study design

Results

Multivariate statistical analysis

The investigation protocol is shown in Figure 1. A total of 183 molecular features were extracted from the serum samples, and multivariate analysis was performed using SIMCA 13.0. The HC and SS samples were clearly separated by PLS-DA; the Q^2 and R^2Y values (R^2Y (for PLS-DA) and Q^2 represent the goodness of fit and prediction ability of the current model, respectively) were 0.95 and 0.96, respectively, which indicated that the model was reliable (Fig. 2A). Moreover, an overfitting analysis (Fig. 2B) showed that the model was appropriate and exhibited no evidence of chance correlation. The features were considered significant (VIP >1.0 and FDR <0.05) and were distinguished from the controls. The heatmap shown in Figure 3C illustrates the cluster identification. Similarly, PLS-DA score plots (Fig. 3A) showed that the patients with SS and HCs can be distinguished by 21 significantly altered metabolites (Table I). The validity of the original model was confirmed by the goodness of fit and prediction (R2 and Q2) of the original PLS-DA model (Fig. 3B). Overall, the serum samples from the patients with SS presented increased levels of alanine, tryptophan, glycolic acid, pelargonic acid, cis-1-2-dihydro-1-2-naphthalenediol, diglycerol, capric acid, turanose, behenic acid, dehydroabietic acid, stearic acid, linoleic acid,

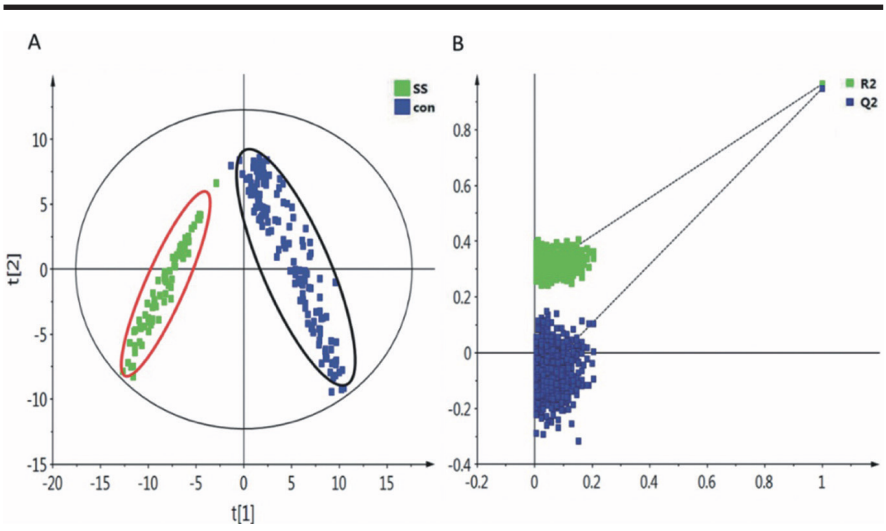

Fig. 2. PLS-DA of serum metabolomic data from patients with SS and HCs in the discovery set. **(A)** PLS-DA of serum metabolomic data from patients with SS and HCs. 183 serum metabolites distinguished patients with SS from HCs. **(B)** Validation plot obtained from 999 random permutation tests showing the robustness of the original PLS-DA model (R2= (0.0, 0.284), Q2= (0.0, -0.105)).

heptadecanoic acid, valine and lactic acid and lowerlevels of catechol, anabasine, 3-6-anhydro-D-galactose, beta-gentiobiose, 2-ketoisocaproic acid and ethanolamine compared with the samples from the HCs.

Validation of potential biomarkers with a test set

To define key metabolites distinguishing patients with SS from HCs, another set of 119 serum samples was collected and analysed for internal validation. After applying the same analytical and statistical methods as for the discovery set, the stability of the selected metabolites was displayed using a distribution chart (Fig. 4). This chart provided additional information, and three metabolites (lactic acid, valine, and heptadecanoic acid) were removed from the group of potential biomarkers due to significant differences between the discovery and validation sets. A total of 18 metabolites were thus retained in the candidate biomarker pool.

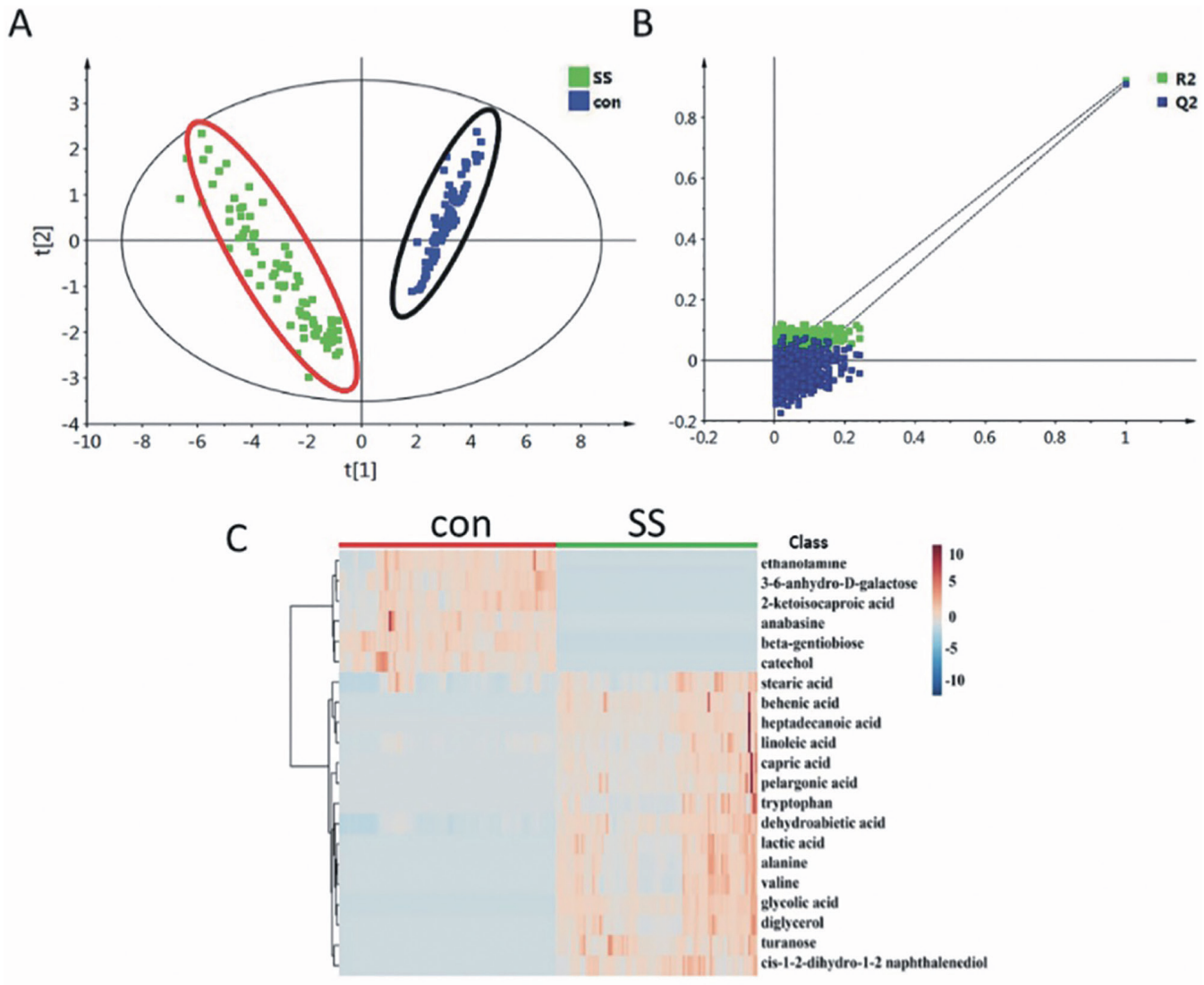

Fig. 3. PLS-DA analysis based on differential metabolites.
A: The 21 significantly altered differential metabolites screened were able to distinguish patients with SS from HCs in the discovery set.
B: Validation model scores obtained using 999 random permutation tests that did not outperform the original PLS-DA model (R2= (0.0, 0.007), Q2= (0.0, -0.092)).
C: Heatmap of altered metabolites between patients with SS and HCs. The red and blue colours indicate increased and decreased levels, respectively. Rows: metabolites; columns: serum samples.

Through forward LR analysis, stearic acid and linoleic acid were identified as reliable metabolites in the regression model, and the diagnostic potential of these two metabolites was evaluated. The results (Fig. 5A) obtained using both sets confirmed that the two metabolites were able to separate patients with SS from HCs with high sensitivity, specificity, and diagnostic performance. Fig. 5B displays the prediction probability values of these two potential biomarkers. A novel serum biomarker model that includes these two metabolites (stearic acid and linoleic acid) was ultimately defined for distinguishing patients with SS from subjects without SS. We collected relevant proteins used to detect patients with SS and analysed the relationship between each metabolite and CRP, ESR, IgG, SSA, SSB, ANA, IgA and RF to determine their clinical relevance. Fig. 5C shows the correlations between each metabolite and these clinical parameters. Six of 21 differentially expressed metabolites were deleted due to lack of sufficient values for the correlation analysis.

Metabolic pathway analysis

Integrated pathway analysis was performed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic library and MetaboAnalyst 3.0 (Montreal, QC, Canada) to map all annotated metabolites into biochemical pathways for advancing mechanistic interpretation. The results were presented in Fig. 6. Based on the obtained results, the most impacted by SS pathways were: linoleic acid metabolism; unsaturated fatty acid biosynthesis; aminoacyl-tRNA biosynthesis; valine, leucine and isoleucine biosynthesis; glycerolipid metabolism; selenocompound metabolism; galactose metabolism; alanine, aspartate and glutamate metabolism; glyoxylate and dicarboxylate metabolism; glycer-

Table I. List of metabolites selected based on the multivariate statistical analysis between patients with SS and HCs in the discovery set.

Metabolite	Average Rt (min)	Quant mass	[a]VIP	[b]*p* value	[c]FDR
Glycolic acid	4.59	147	1.875	<0.001	<0.001
Beta-gentiobiose	14.15	204	1.773	<0.001	<0.001
Turanose 2	12.2	73	1.680	<0.001	<0.001
Dehydroabietic acid	12.24	239	1.670	<0.001	<0.001
Cis-1-2-dihydro-1-2-naphthalenediol	7.66	191	1.658	<0.001	<0.001
Diglycerol	7.05	103	1.576	<0.001	<0.001
Lactic acid	4.46	147	1.546	<0.001	<0.001
Alanine	4.8	116	1.465	<0.001	<0.001
Valine	5.67	144	1.457	<0.001	<0.001
Behenic acid	13.08	117	1.433	<0.001	<0.001
3-6-anhydro-D-galactose	9.52	231	1.373	<0.001	<0.001
2-ketoisocaproic acid	5.7	207	1.353	<0.001	<0.001
Catechol	6.44	254	1.310	<0.001	<0.001
Heptadecanoic acid	11.04	117	1.289	<0.001	<0.001
Ethanolamine	6.07	201	1.284	<0.001	<0.001
Capric acid	7.37	117	1.274	<0.001	<0.001
Stearic acid	11.47	117	1.127	<0.001	<0.001
(+/-) anabasine 1	7	239	1.114	<0.001	<0.001
Pelargonic acid	6.74	215	1.058	<0.001	<0.001
Tryptophan	11.5	202	1.007	<0.001	<0.001
Linoleic acid	11.37	81	1.004	<0.001	<0.001

[a]VIP was obtained from the OPLS-DA model with a threshold of 1.0. [b]*p*-values were obtained from one-way ANOVA. The value of [c]FDR was obtained from the adjusted *p*-value after FDR correction using the Benjamini-Hochberg method.

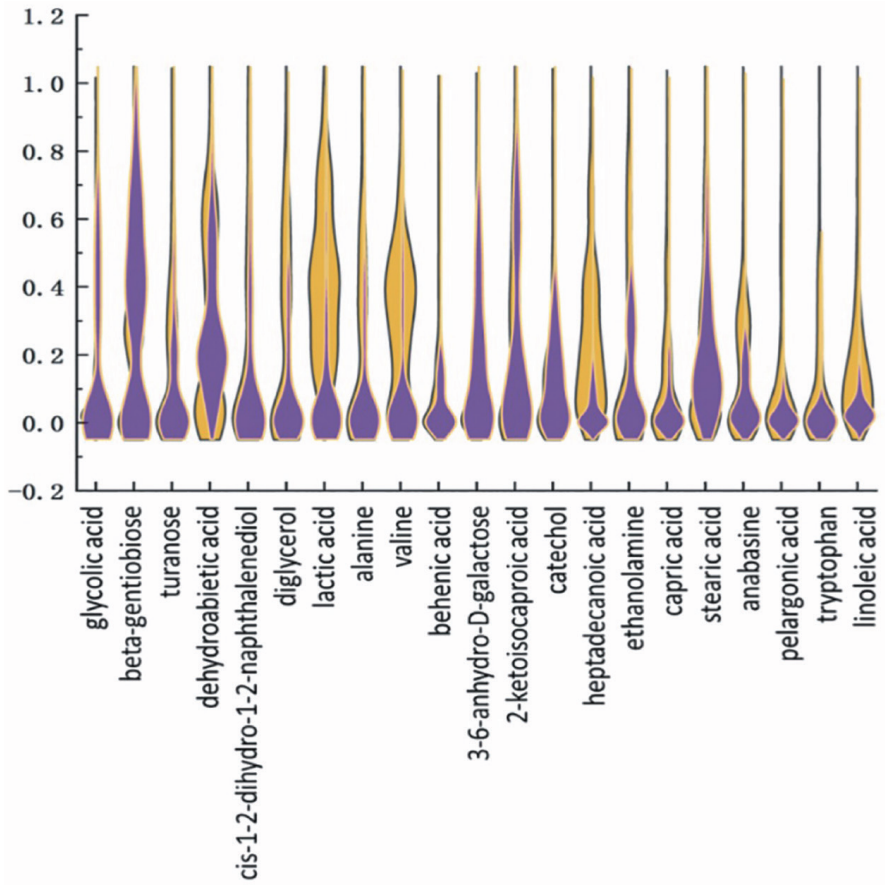

Fig. 4. Distribution chart comparing each metabolite between the discovery (purple) and validation datasets (yellow).

ophospholipid metabolism; and valine, leucine and isoleucine degradation. It suggested that aetiology and processes linked to SS were complicated and associated with many factors, the biosynthesis of unsaturated fatty acids and linoleic acid metabolism were considered the two most important factors.

Discussion

In this study, we evaluated whether an examination of serum metabolomics can increase the value of predicting SS and ultimately be useful in the clinic. The 21 key metabolites that exhibited the same trend in both the discovery and validation sets might provide clues to the exploration of potential biochemical pathways in patients with SS. We then calculated the Pearson correlation coefficient for these differential substances and clinical indicators with diagnostic significance and found a weak correlation (Fig. 5C); it was difficult to prove that this finding was directly related to the clinic. Although the information obtained from metabolomics studies might be of some help to obtaining a better pathophysiological understanding of the disease, the biological interpretation of these data remains difficult.

Previous studies have demonstrated that alterations in the plasma levels of unsaturated fatty acids (UFAs) are involved in SS. For example, linolenic acid appears to be upregulated in plasma samples from patients with SS (8). Linoleic acid can be elongated after dehydrogenation and desaturated during the synthesis of arachidonic acid (ARA). ARA in turn plays an important role as a membrane phospholipid fatty acid in cells involved in the inflammatory response and is also a direct precursor to biologically active lipid mediators such as prostaglandins and leukotrienes. However, these substances can cause chronic diseases such as inflammation and cancer if produced in excess (20).

Additionally, we observed significantly higher levels of stearic acid in the SS group than in the HCs. The study conducted by Tsoukalas *et al*. (21) revealed that the metabolism of total fatty acids (TFAs) in serum might play a role in the development and progression of autoimmune diseases (ADs) and can help

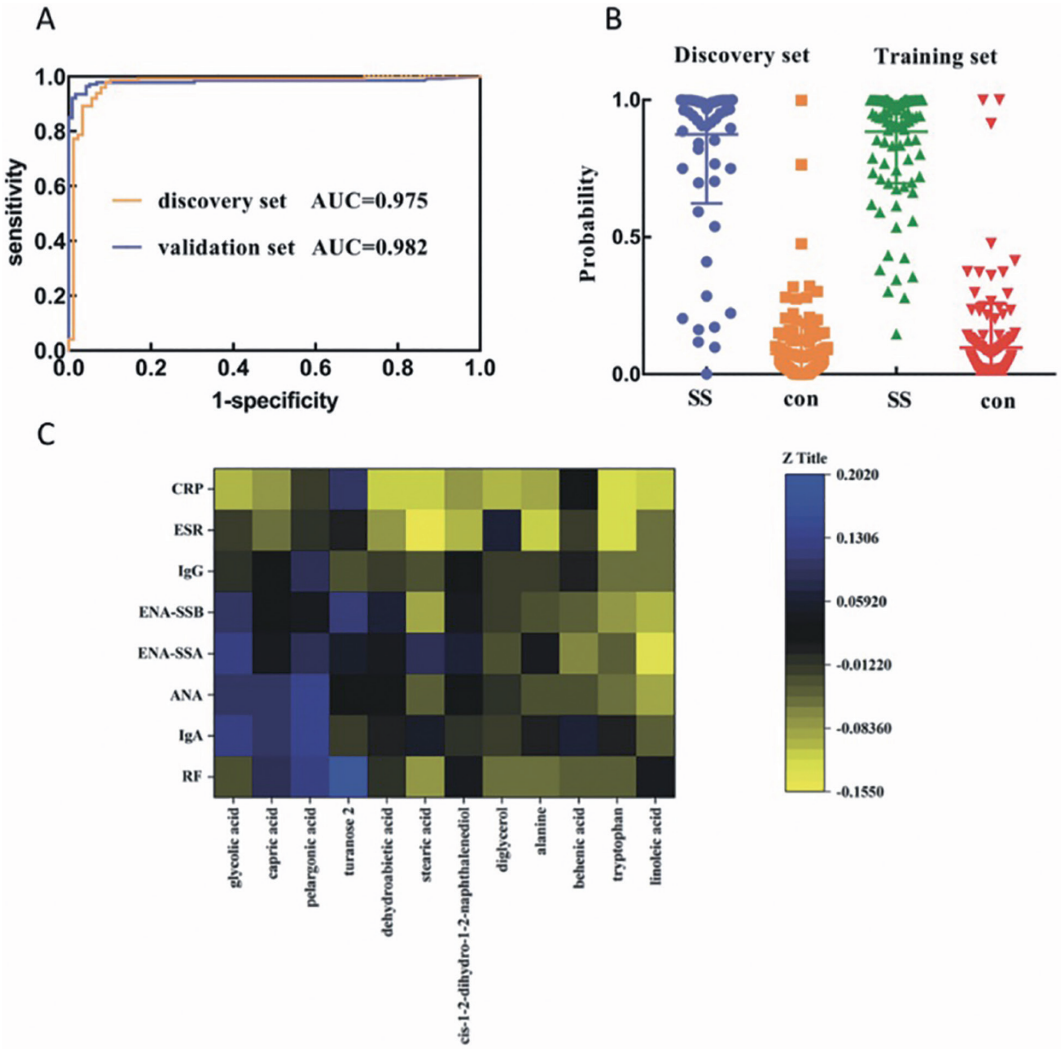

Fig. 5. Evaluation of the diagnostic potential of differentially expressed metabolites.
A: ROC curves for the combination of two potential biomarkers (stearic acid and linoleic acid).
B: Discrimination of patients with SS from healthy individuals using the combination of the two potential biomarkers.
C: Heat map of the Pearson correlation coefficients between the contents of the differentially expressed metabolites and clinical parameters. The shades of each colour represent the strength of the relationship, and the blue, black, and yellow colours represent positive, no, and negative correlations, respectively.

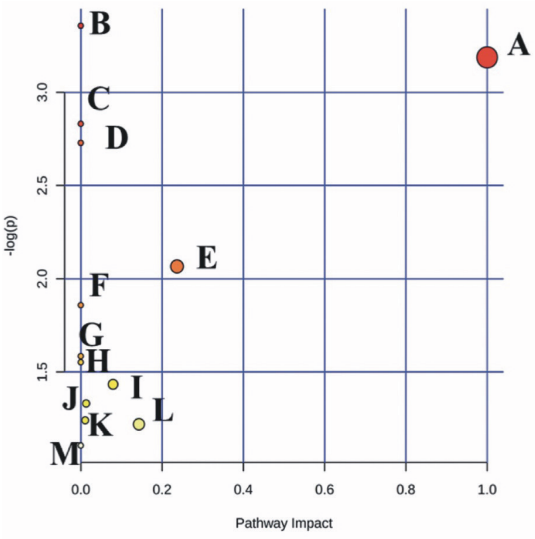

Fig. 6. Pathway analysis of the altered metabolites identified in patients with SS compared with HCs. **A**. Linoleic acid metabolism; **B**. biosynthesis of UFAs; **C**. aminoacyl-tRNA biosynthesis; **D**. valine, leucine and isoleucine biosynthesis; **E**. glycerolipid metabolism; **F**. selenocompound metabolism; **G**. galactose metabolism; **H**. alanine, aspartate and glutamate metabolism; **I**. glyoxylate and dicarboxylate metabolism; **J**. glycerophospholipid metabolism; **K**. valine, leucine and isoleucine degradation.
"Pathway impact" is a weight calculation based on topology analysis. And was calculated by adding up the importance measures of each of the matched metabolites and then dividing by the sum of the importance measures of all metabolites in each pathway.

with the early diagnosis of ADs. We found that stearic acid is involved in the synthesis of plasmalogen and in the β-oxidation of long-chain saturated fatty acids in mitochondria. Plasmalogens are a subclass of glycerophospholipids that contain vinyl ether, play multiple roles in cell function and form part of the cell membrane (22). Fatty acids are used as substrates to generate acetyl-coA by mitochondrial β-oxidation, with NADH and FADH2. Furthermore, fatty acids can act as a source of energy for mitochondria and provide approximately 80% of the ATP required by the liver and heart. Fatty acid oxidation disorders usually cause cardiomyopathy, liver disease, hypoglycemia, and skeletal muscle weakness, among other effects (23). Previous studies have suggested that these two metabolic pathways may contribute to the development of SS.
Our findings are consistent with previous findings that tryptophan metabolism regulates the immune response in primary SS (8). Several amino acids were found to be slightly changed in patients with SS, for example, the serum capric acid level in patients with SS was significantly higher than that in HCs. Capric acid is a medium-chain fatty acid found in coconut oil and can act as a reinforcing agent to boost the immune system.

Moreover, the polyphenols, lauric acid and capric acid in coconut oil are often recommended as treatments for osteoarthritis and rheumatoid arthritis (24). In addition, the RT-PCR results obtained by Kim E *et al*. (25) showed that dehydroabietic acid significantly reduces nitric oxide (NO) production and inhibits the expression of inflammatory genes.

One study had same limitations. First, we lacked SS patients' BMI, duration of disease and medical history, etc. Second, target-metabolomics need to be done after untarget-metabolomics. In the future, more clinical characterisation of the individuals suffering of SS will be collected, and target-metabolomics will be further confirmed the metabolites found.

Conclusion

In this study, a GC-MS-based metabolomics approach was employed to compare the metabolic characteristics between patients with SS and HCs. We observed a number of important metabolites that were altered in patients with SS, and these metabolites were primarily associated with the metabolism of unsaturated fatty acids, the synthesis of plasma and the mitochondrial beta-oxidation of long-chain saturated fatty acids. Our findings demonstrate that a metabolomic approach is a promising tool for exploring the complex metabolic states of diseases.

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