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Serum metabolic profiling of abnormal savda by liquid chromatography/mass spectrometry☆

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ABSTRACT

Abnormal savda is a special symptom in Uigur medicine. The understanding of its metabolic origins is of great importance for the subsequent treatment. Here, a metabonomic study of this symptom was carried out using LC–MS based human serum metabolic profiling. Orthogonal signal correction partial least-squares discriminant analysis (OSC-PLS-DA) was used for the classification and prediction of abnormal savda. Potential biomarkers from metabonomics were also identified for a metabolic understanding of abnormal savda. As a result, our OSC-PLS-DA model had a satisfactory ability for separation and prediction of abnormal savda. The potential biomarkers including bilirubin, bile acids, tryptophan, phenylalanine and lyso-phosphatidylcholines indicated that abnormal savda could be related to some abnormal metabolisms within the body, including energy metabolism, absorption of nutrition, metabolism of lecithin on cell membrane, etc. To the best of our knowledge, this is the first study of abnormal savda based on serum metabolic profiling. The LC/MS-based metabonomic platform could be a powerful tool for the classification of symptoms and for the development of this traditional medicine into an evidence-based one.

1. Introduction

The study of systems biology gives a holistic understanding of the organism. As an important component of systems biology, metabonomics can be defined as an attempt to measure all the changes in the metabolites that are present within a cell, tissue or organism during a genetic modification or physiological stimulus [1,2]. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)-based technologies are generally used for the metabolic profiling of the biological matrices [3–5]. Combined with chemometric data-analysis techniques, the spectra generated from NMR or MS can be applied to establish patterns for investigating responses of physiological or pathological stressors. Global metabolites profiling of biofluids can be used to probe the state of the organism not only to give a systemic view of the final endpoints of regulatory physiological progress, but also to provide a powerful tool for the prognosis and diagnosis of diseases [5–7].

Compared with NMR, MS is a more sensitive technique. When coupled to liquid chromatography (LC), it allows higher resolution and sensitivity for the low abundance metabolites. High-performance liquid chromatography (HPLC) coupled to mass spectrometry is now increasingly being used in the metabolic profiling of biological fluids [5,8–10]. To obtain a higher throughput and more comprehensive profiles, new LC systems and columns with sub-2 μm particle stationary phases have arisen and shown their values in the metabolic profiling studies. Ultra performance LC is one of these techniques and has been used in many metabonomics investigations [11,12].

Abnormal savda is a special state and symptom in Uigur medicine [13]. This abnormal state is due to the unregulated metabolism of the body, thus causes the dysfunction of the organism. It is considered as the one of the origins of many diseases, such as tumors, asthma, diabetes, hypertension and so on [14–16] and has been reported to be related to the damage induced by oxidative stress and free radicals [17,18]. The treatments for abnormal savda are considered as a complimentary method and have also shown their effects through the millennia of clinical practices [13–18]. Munziq and Mushil are traditional Uighur herbal medicines for abnormal savda, which could have antioxidant properties protecting mitochondria against oxidative damage [18]. Study of abnormal savda would be of great value not only for the investigation of

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the symptom, but also for the understanding of the basis of Uigur medicine.

In the present study, ultra performance liquid chromatography coupled to Q-TOF mass spectrometry (UPLC/Q-TOF MS) system was used for the metabolic profiling of human serum. By using chemometric analysis such as principal component analysis (PCA), we investigated the substance basis of abnormal savda. Furthermore global metabolite profiling was used to study whether changes in the metabolite profile were associated with changes in physiopathology (e.g. clinical evidence).

2. Materials and methods

2.1. Chemicals

Acetonitrile and formic acid (HPLC grade) were purchased from Merck (USA), distilled water was filtered through a Milli-Q system (Millipore, MA). Leucine–enkephalin was from Sigma–Aldrich (USA).

2.2. Sample collection and preparation

The study was approved by the Ethics Committee of Xinjiang medical university. Since abnormal savda is common in many diseases, patients with five different diseases including bronchial asthma, coronary heart disease, diabetes, gastritis and chronic renal failure were enrolled in the study. There were 110 patients with clear diagnosis and 20 healthy volunteers enrolled in this study (Table 1). With the permission, serum samples from fasting individuals were collected before breakfast and stored at −80 °C until analysis. Abnormal savda was diagnosed by three Uigur doctors with at least 5 years of clinical experience. Blood samples were collected for both clinical examination (patients) and metabolic profiling. Routine clinical parameters were measured including counting of blood cell (erythrocyte, acidophil leukocyte, basophilic leukocyte, neutrophil leukocyte, mononuclear leukocyte, lymphocyte and platelet), hemoglobin, enzymes (ALT, AST, γ-GT GGT and ALP), total proteins, albumin, globulin, total bilirubin, direct and indirect bilirubin, total bile acids, creatinine, uric acid, urea nitrogen, glucose, and ions (K^+, Na^+, Ca^{2+}, $Mg^{2+}$and Cl^-).

Samples were allowed to thaw prior to analysis. A standard sample was prepared by mixing 10 μL of each sample [19], then acetonitrile (600 μL) was added to serum (150 μL). The mixture was shaken vigorously for 30 s, then was laid at 4 °C for 5 min, centrifuged at 15000 × *g* for 10 min at 4 °C. The supernatant (650 μL) was lyophilized.

2.3. Chromatography

Chromatographic separation was performed on a 10 cm × 2.1 mm, 1.7 μm BEH C18 column (Waters, USA) using a ultra performance liquid chromatography system (Waters, USA). The column was maintained at 35 °C. Mobile phases A and B consisted of 0.1% aqueous formic acid and acetonitrile, respectively. The elution followed a linear gradient of 2–100% B in 35 min at a flow rate of 0.35 mL/min. The lyophilized serum specimens were diluted in 100 μL solvent consisting of 1:4 distilled water and acetonitrile, a 4 μL injection of each sample was made onto the column. The standard sample ran six times continuously for the study of the stability of the method. All the samples were kept at 4 °C during the analysis and analyzed once at a random order.

Table 1
Diagnosis of patients in the study

	Abnormal savda	Others
Bronchial asthma	14	34
Coronary heart disease	10	6
Diabetes II	9	7
Gastritis	14	1
Chronic renal failure	9	4
Total	58	52

2.4. Mass spectrometry

A Waters Q-TOF micro-MS system (Waters MS Technologies, Manchester, UK) operated in positive ionization modes was used in this study. Nitrogen was used as nebulization gas and was set to 500 L/h at a temperature of 300 °C, the cone gas (nitrogen) was set to 50 L/h and the source temperature was at 100 °C. A capillary voltage and a cone voltage were set to 3100 and 35 V, respectively. The MCP detector voltage was set to 2600 V. The Q-TOF micro-MS acquisition rate was set to 0.48 s with a 0.1 s interscan delay. Tune page was used to regulate the sample cone voltage. Argon was employed as the collision gas. Scan range was from 100 to 1000 *m*/*z*. Data was collected in centroid mode. Target MS/MS analysis was used for the identification of the potential biomarkers. The collision energy was set as 10, 20 and 35 eV, respectively.

The lock spray was used to ensure mass accuracy and reproducibility; leucine–enkephalin was used as the lock mass at a concentration of 2 ng/μL and flow rate 10 μL/min. The lock spray frequency was set at 20 s.

2.5. Data analysis

The raw data were analyzed by MarkerLynx (Waters, UK) for peak detection and alignment. The parameters were set as follows, the mass tolerance was set at 0.5 amu, and peak width at 5% height and peak-to-peak baseline noise were calculated automatically by the software. The mass window was set at 0.5 amu and retention time window was set at 0.2 min. The noise elimination was level 5. The minimum intensity was set at 1%. Then the raw data were exported for PCA and orthogonal signal correction partial least-squares discriminant analysis (OSC-PLS-DA) using SIMCA-P 11.0 (Umetrics AB, Umeå, Sweden). Areas of all the peaks were normalized to 10000 per run, then the normalized and mean-centered

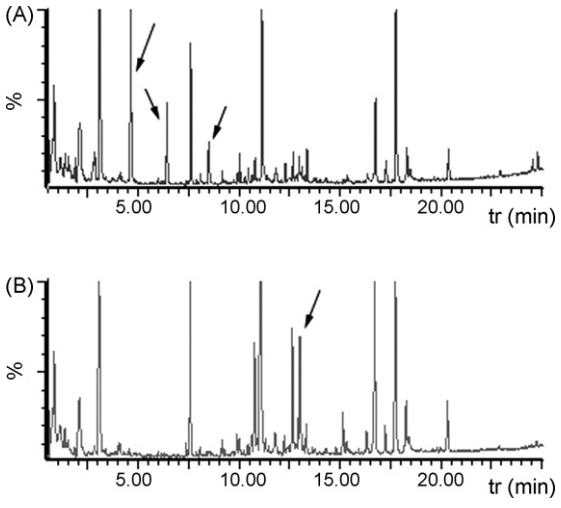

Fig. 1. Typical serum UPLC/Q-TOF TIC (total ion current) chromatograms from bronchial asthma patients with abnormal savda (A) and healthy controls (B). Peaks marked with arrows were some of the distinct variances between abnormal savda and healthy controls.

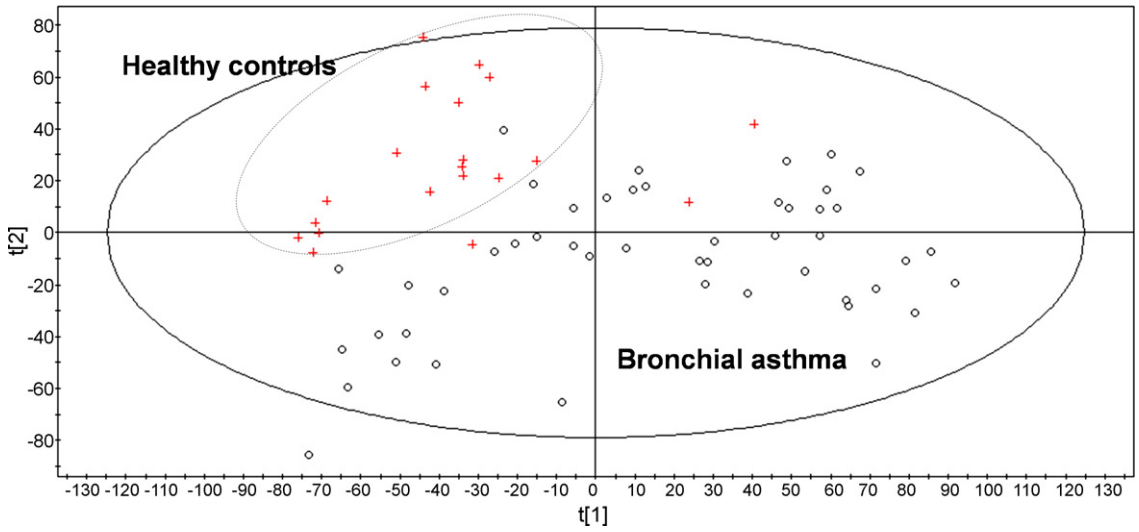

Fig. 2. PCA score plot of bronchial asthma patients (o) and the healthy controls (+).

data were pareto-scaled before analysis. The OSC filter removed the uncorrelated signals resulting in information of the within-class variation. Parameters of PLS-DA such as R^2Y and Q^2 were used in the study for the evaluation of the models indicating the goodness of fit and the ability of prediction. Response permutation test was used to assess a model to avoid over-fitting due to chance correlation.

3. Results and discussion

3.1. Establishment of metabonomics analysis method

In Uigur medicine abnormal savda is commonly considered as an abnormal state of the body, it can be found in patients of nearly all diseases, and its correct diagnosis is of great values for the treatment of diseases and the development of drugs. There are also many literature reports which have described the relationship between abnormal savda and oxidative damage, immune state and so on [13–18].

In order to understand abnormal savda by metabolic profiling, we need a metabonomics method including the collection of data on metabolites by UPLC/Q-TOF and data analysis by multivariable statistical software. Patients with a single disease were preferred for the establishment of such a metabonomic investigation. Bronchial asthma patients were enrolled as they have a larger number in the hospital and relatively less accompanying symptoms.

Serum metabolic profiling was performed and a typical UPLC/Q-TOF TIC (total ion current) chromatography was presented in Fig. 1. The analysis of the standard sample showed that the relative standard deviations of retention time were below 0.5%, the relative standard deviations of area and peak height were from 2 to 10%. The results showed that this method had good repeatability for metabolite analysis.

It can be seen from Fig. 1, that some of the metabolic changes in patients could be found directly in the TIC chromatograms (peaks marked with arrows). In order to gain a comprehensive view of the metabonome, PCA was used in the subsequent data analysis. The PCA score plot of bronchial asthma patients and healthy controls was shown in Fig. 2. Separation between patients and controls was clear using the first two components. These two components explained 55% of the total variances. This unsupervised analysis of PCA offered a trend of the metabolic changes between the bronchial asthma patients and controls. These results show that the metabolic profiling method established could be further used for classifying and understanding abnormal savda.

Table 2
Potential biomarkers of abnormal savda

Retention time	*m/z*	VIP	Metabolites	Levels in abnormal savda	Related pathway
18.25	496.32	9.85	LPC C16:0	↓	Lecithin metabolism
20.79	524.34	8.41	LPC C18:0	↓	Lecithin metabolism
17.28	520.3	4.98	LPC C18:2	↓	Lecithin metabolism
18.24	991.66	3.38	LPC C16:0	↓	Lecithin metabolism
11.24	585.25	3.05	Bilirubin[a]	↑	HEME degradation
3.11	145.93	2.76	Tryptophan fragment	↓	Precursor of serotonin, essential amino acid
22.41	256.17	2.32	Unidentified	↑	
2.13	119.95	2.29	Phenylalanine fragment	↓	Precursor of catecholamine, essential amino acid
3.11	187.98	1.98	Tryptophan fragment	↓	Precursor of serotonin, essential amino acid
18.83	522.33	1.67	LPC 18:1	↓	Lecithin metabolism
13.18	414.29	1.65	GCDCA	↑	Absorption of fats, cholesterol metabolism
15.38	357.27	1.34	Unidentified	↑	
0.71	203.02	1.06	Unidentified	↓	
10.97	181.01	1.06	Unidentified	↑	

LPC: lysophosphatidylcholines; GCDCA: glycochenodeoxycholic acid. The levels of potential biomarkers in abnormal savda compared with other patients were marked with (↑): upregulated and (↓): downregulated.

[a] Have not been confirmed by standard sample.

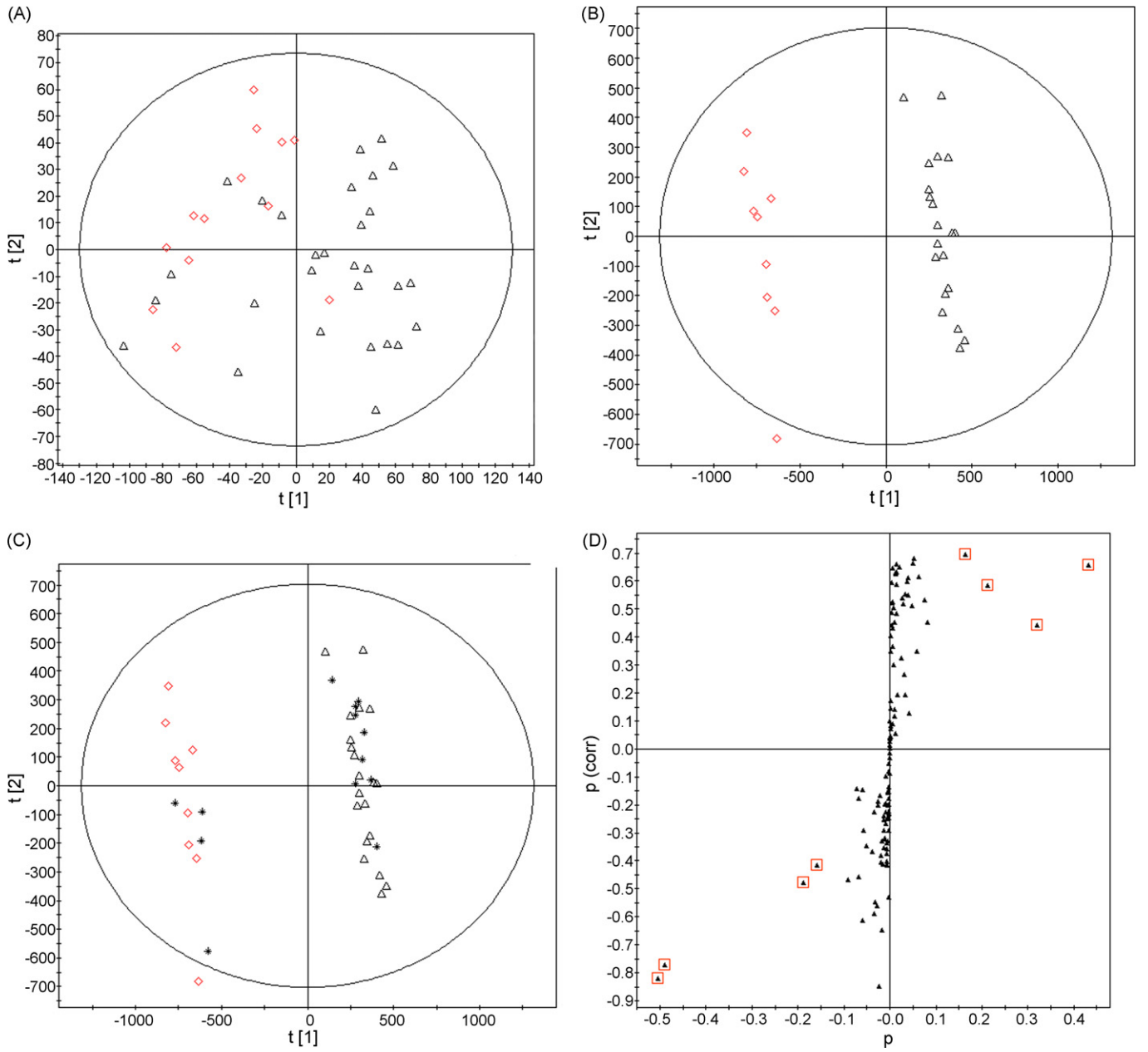

Fig. 3. (A) PCA score plot of the bronchial asthma patients, (B) OSC-PLS-DA score plot of abnormal savda patients and other patients and (C) *T* predicted score plot of OSC-PLS-DA. (◇) abnormal savda patients, (△) other patients and (*) predicted samples. (D) *S*-plot of OSC-PLS-DA. Metabolites in the (□) could be selected as potential biomarkers.

3.2. Separation of abnormal savda from other bronchial asthma patients

PCA analysis was performed among all the bronchial asthma patients with different symptoms of abnormal state. Four components were calculated and explained 60% of the total variances. Fig. 3A shows the PCA score plot of the patients. The Hotelling's T^2 range was used to provide the number of the outliers among the samples at the level of 0.05. A trend of separation between abnormal savda and other abnormal states could be seen from Fig. 3A. But the result was still not satisfactory. In order to get a better separation, OSC-PLS-DA analysis was performed. Data from 9 abnormal savda patients and 21 other patients were used as the training set. The remaining patients comprised the prediction set. Four components were calculated and the corresponding model parameters denoting interpretability and prediction, R^2Y and Q^2 were 0.99 and 0.98, respectively. No over-fitting was found according to the permutation validation. A clear separation could be seen in Fig. 3B. The value of Q^2 showed an encouraging prediction ability of this OSC-PLS-DA model. Then the model was used for the subsequent prediction. The *T* predicted score plot is shown in Fig. 3C. All of 13 samples are well predicted. The results indicated that the OSC-PLS-DA model had a good capability for the classification or diagnosis of abnormal savda from other bronchial asthma patients. The possible metabolic differences between abnormal savda and other abnormal states were clearly demonstrated.

3.3. Understanding of the metabolic origin of abnormal savda

In order to get an insight into the metabolic changes among abnormal savda patients, we tried to find the related potential biomarkers based on the former OSC-PLS-DA analysis. These

metabolites were related to the difference between abnormal savda and other abnormal states. The jack-knifed confidence interval was also referred (Fig. 3D). The *S*-plot showed the combination of covariance and correlation information and avoided the increases of the risk for false positives (type I error) [20]. The value of variable importance in the projection (VIP) was used in the selection (VIP > 1, isotopes omitted, Table 2). After that, the potential biomarkers were identified according to the methods and results of our former work [21–23]. The website of Pubchem (http://pubchem.ncbi.nlm.nih.gov) and mass bank (http://www.massbank.jp) were searched for the metabolites and their standard MS/MS spectra. And standard samples were used for the confirmation of other compounds. The final results of the selection and identification of potential biomarkers are given in Table 2.

Glycochenodeoxycholic acid and bilirubin were selected as potential biomarkers in the study. These metabolites may play important roles in the metabolic changes of abnormal savda. Bile acids are synthesized from cholesterol in the liver and are crucial for the absorption of dietary fats and lipid-soluble vitamins in the intestine [24]. And profiles of bile acids are also indicative of various diseases [24]. Adequate absorption and metabolism are essential for the homeostatic regulation of the body. Bile acids are also related to the metabolism of fatty acids and consumption of glucose in the liver [25]. And bile acids are also reported as regulators for energy metabolism of the body [26]. Tryptophan and phenylalanine are essential amino acids which cannot be synthesized by the body. They are either incorporated into proteins or they are broken down for energy and metabolic intermediates. Tryptophan is the precursor of serotonin, which is an important neurotransmitter, and phenylalanine is also precursor of another important neurotransmitter, catecholamine [25]. The lack of these two amino acids may also indicate the loss of the balance between nutrition intake and consumption. The increase of energy and protein expenditure could be the main reasons for the decrease of these two amino acids. And these above metabolites may indicate the changes in these metabolites' concentrations and a lack of some essential nutrients can be important metabolic changes in abnormal savda patients. Lyso-phosphatidylcholines are metabolites of membrane phosphatidylcholines, and the other product of phosphatidylcholine degradation is arachidonic acid, which is the substrate of many biologically active compounds. The metabolites of arachidonic acid such as prostaglandins, thromboxanes, and leukotrienes are mediators and regulators of many progress including inflammation and immune function [25]. Since oxidative stress had been reported to be related to abnormal savda, it could be the stimuli that activates the metabolism of phosphatidylcholines and causes the down-regulation of lyso-phosphatidylcholines [25].

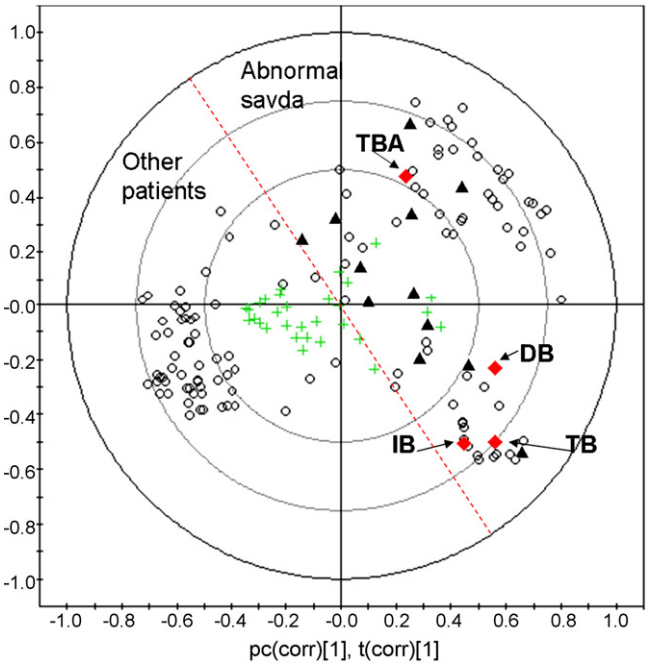

Fig. 4. PLS bi-loading plot of bronchial asthma patients. The plot shows the correlation among the patients with the diagnosis of abnormal savda (▲), other patients (✚), LC/MS metabolites (o) and clinical examination (◆). The red dot line on the plot shows the separation between abnormal savda patients and other patients. TBA: total bile acids, DB: direct bilirubin, TB: total bilirubin and IB: indirect bilirubin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

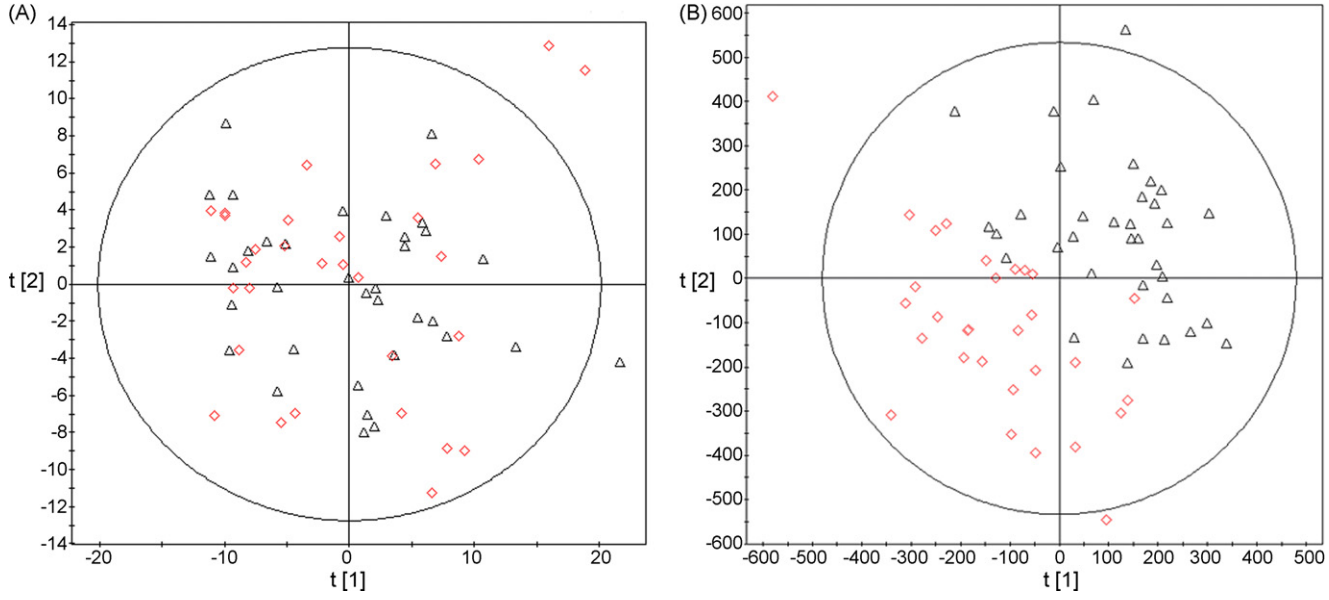

Fig. 5. (A) PCA score plot of all the patients. Abnormal savda patients (△) cannot be separated from other patients (◇) and (B) OSC-PLS-DA score plot of all the patients, abnormal savda patients (△) and other patients with one of five diseases (◇) can be separated clearly.

To gain a possible relationship between metabolic profiling and the clinical parameters, a PLS analysis was then performed. The X-matrix consisted of the LC/MS data while the Y-matrix consisted of the clinical parameters from the hospital. Correlation coefficient between clinical examination and the LC/MS derived metabolites were calculated by PLS. The PLS bi-loading plot (correlation scaled) showed the relationships between patients, metabolites and the clinical parameters (Fig. 4). Of all the clinical examinations given in Section 2.2, only bilirubins (direct and indirect bilirubins) and total bile acids had significant correlations with the detected metabolites. And they also showed a close correlation with abnormal savda in the plot. However, these clinical parameters themselves could neither separate nor show significant probability of the abnormal savda. According to this result, we confirmed our presumption that abnormal savda were correlated with the state of hepatic metabolism, which could be presented from both metabolic profiling and the clinical parameters. However, the metabolic profiling had also proved to be a powerful tool for the presentation of metabolic patterns.

3.4. Extended study of abnormal savda in different diseases

In order to get further knowledge of abnormal savda in different diseases, patients with one of five different kinds of chronic diseases were enrolled and separated into two classes (Table 1). PCA analysis was performed. Fig. 5A shows the PCA score plot, it is seen that the classification between abnormal savda and other abnormal states is not clear. The OSC-PLS-DA was performed subsequently. After the uncorrelated signals were removed by OSC filtering a clear classification could be seen from the OSC-PLS-DA score plot (Fig. 5B). Three components were calculated, $R^2Y = 0.79$ and $Q^2 = 0.58$ were achieved, the response permutation test shows no over-fitting was found. Variables which had been selected as biomarkers were similar to those in Table 2. From the results it could be presumed that abnormal savda in different diseases has the same substance basis or characteristic metabolic profiles. So abnormal savda patients could be separated from others and treated in the clinic. However, these metabolic variations caused by abnormal savda were smaller than those caused by different chronic diseases. This phenomena may indicate that the therapy for abnormal savda could be a personalized and complementary method for the treatment of these diseases.

4. Conclusions

In this study, we reported an investigation of the abnormal savda, a symptom in Uigur medicine, using the method of serum metabolic profiling and chemometric analysis. The results show metabolic profiling could find out the latent variances and are able to define chemometric models for the prediction of abnormal savda. The potential biomarkers could also be helpful for the understanding of abnormal savda. It was found from our metabonomics study that abnormal savda might be related to energy metabolism, absorption of nutrients, metabolism of lecithin on cell membrane, and has a characteristic metabolic profile. These results strongly suggested that serum metabolic profiling based on LC/MS could be a powerful platform for understanding substance basis of the traditional medicine diagnosis. Combined with the interpretation of the related biomarkers, the platform would also probe into the physiopathological origins as a top-down system biological tool.

Acknowledgments

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