REVIEW ARTICLE

Syndrome differentiation of chronic hepatitis B patients by integrating routine clinical laboratory and plasma metabolomics analysis data

Authors

Peng Gao[1,2,3,4,5*], Xin Huang[1], Aijun Sun[1,2], Jinlong Guo[6], Hangyu Gong[1,6], Sichao Li[7], Lu Xing[7*]

Affiliations

1. Translational Medicine Center, Dalian Sixth People's Hospital. Dalian, China.

2. Dalian Municipal Clinical Research Center for Viral Hepatitis, Dalian, China

3. Clinical Laboratory, Dalian Sixth People's Hospital. Dalian, China,

4. Key Laboratory of Liaoning Tumor Clinical Metabolomics (KLLTCM), Jinzhou, Liaoning, China

5.   Personalized Treatment and Diagnosis Research Center, The First Affiliated Hospital of Jinzhou Medical University and Dalian Institute of Chemical Physics, Chinese Academy of Sciences

6. Liver Disease Department, Dalian Sixth People's Hospital. Dalian, China.

7. Traditional Chinese Medicine Department, Dalian Sixth People's Hospital. Dalian, China.

*Corresponding authors:

1. Prof. Dr. Peng Gao

Email: gaop@dicp.ac.cn

2. Lu Xing

Email: xingluhao@126.com

ABSTRACT

Purpose: Traditional Chinese Medicines (TCMs) has been officially approved for chronic hepatitis B infection treatment in China. Correct syndrome differentiation is the key prerequisite for rational prescribing TCMs. Unfortunately, TCM physicians have scarcities of objective measures for syndrome differentiation and their diagnosis exclusively relies on individual experience. This study aimed to find out some objective parameters to aid syndrome differentiation.

Methods: Three commonly encountered clinical syndromes named accumulated dampness-heat syndrome (ADHS), spleen deficiency with liver depression (SDLD) and blood stasis vessel obstruction syndrome (BSVO) were selected.   64 qualified patients with definite syndromes were enrolled and their plasma amino acids and lipids were profiled by metabolomics analysis. Additionally, their routine clinical laboratory parameters were also collected.

Results: Through orthogonal partial least squares-discriminant analysis, the three syndromes could be properly differentiated. The differential parameters between every two syndromes were screened out. Most of them were of the small molecular metabolites. A distinct difference was found between ADHS and the other two syndromes. BSVO and SDLD showed relatively less discrepancy. Furthermore, it was deduced that sphingomyelin metabolism might dominate ADHS phenotype, and platelet functions might affect SDLD phenotype to some extent.

Conclusions: These findings provided primary evidence for the objective classification of varied syndromes of chronic hepatitis B patients and proved that metabolomics analysis might be a valuable tool to aid syndrome differentiation in some circumstances.

Key words: Metabolomics; Hepatitis B; Traditional Chinese Medicine; Sphingomyelin

1. Introduction

Viral hepatitis is still a threat to public health worldwide. Taking the hepatitis B virus (HBV) infection as an example, globally, it was estimated that about 257 million people were HBV surface antigen positive [1]. In China, hepatitis is mainly caused by HBV infection. The prevalence of HBV infection in China was about 7% and 22% of the patients would progress to chronic hepatitis [2]. Although the incidence is decreasing now, the heavy social and economic burdens of chronic HBV infection are still noteworthy[3, 4].

Currently, pegylated interferon and nucleos(t)ide analogs are the most popular Western Medicines for HBV infection treatment[1]. The former can help to

modulate the patients' immunological responses and the latter can inhibit virus replication. Great efforts have been made to speed up new drug development, but the commercially available anti-HBV medicines are still limited [5]. What makes the status worse is that the frequent mutations of the virus usually result in antiviral therapy using nucleos(t)ide failure [6]. Thus, chronic HBV infection is somehow an untreatable disease in a long time [7].

Traditional Chinese Medicines (TCMs) have a history of nearly 2000 years. Approximately, 0.15 billion patients were given Chinese herbal medicines when they received Western Medicine prescriptions clinically [8]. In China, herbal medicines have been recognized as valuable adjunct measures for HBV infection treatment. As much as 80% of the HBV infected patients had the history of herbal medicines administration and the therapeutic effects were encouraging [5].

The prerequisite for rational TCMs administration is the syndrome differentiation. Spleen deficiency (SD) is the most commonly encountered syndrome in Chinese HBV- infection patients [9]. Notably, patients infected by HBV do not show single syndrome but complex syndromes concomitantly. For example, syndromes of liver depression, damp heat and blood stasis can be often perceived in SD patients [9]. Epidemic studies indicated that the most common complex syndromes in HBV- infection patients were accumulated dampness-heat syndrome

(ADHS), spleen deficiency with liver depression (SDLD) and blood stasis vessel obstruction syndrome (BSVO) [10, 11]. The complex syndrome phenotypes pose a great challenge to differential diagnosis. The reason is that patients with complex syndromes usually exhibit some shared TCM features, such as similar symptoms, signs, pulse characteristics and so on [9].

Disease diagnosis in Western Medicine world is based on varied kinds of objective parameters, such as those of serum tests, image modalities, and physical characteristics. On the contrary, TCM syndrome differentiation is solely dependent on the physicians' subjective judgment, such as those of looking, listening, inquiring and pulse feeling. In other words, correct syndrome differentiation is dominated by the physicians' experience exclusively. Attempts had been made to utilize routine clinical laboratory parameters to help syndrome differentiation. The results were limited. Taking the differentiation of ADHS and SDLD as an example, the area under the receiver operating characteristic curve was only about 0.42-0.62 [10].

Metabolomics is a newly coined omics concept. By using different analytical strategies, metabolomics can be employed to reflect the phenotype properties through quantification as more metabolites as possible in a given biological sample [12]. In the life science arena, metabolomics analysis has exhibited its unique advantages in disease stratification, new biomarker discovery and disease prognosis [12-15].

In this study, a quantitative metabolomics analysis was employed to profile the amino acids and lipids in plasma collected from chronic HBV infection patients. To pursue better differentiation amid the ADHS, SDLD and BSVO groups, routine clinical parameters were also included. Thus, the clinical blood macromolecular test results and the small molecular metabolites quantitation data were combined used in this study. The aims of the study were: 1) to confirm whether there was an objective difference amid the three complex syndrome groups; 2) if the syndrome-related differential parameters could be explained by their reported biological context. The answers to the two questions might help the unexperienced physicians to correctly subtype the chronic HBV infection patients and to prescribe patient-specifically.

2. Materials and methods

2.1 Patients

All the patients were enrolled from Dalian Sixth People's Hospital. For symptom confirmation, every patient was evaluated by three senior TCM physicians independently based on Guidelines for Traditional Chinese Medical Diagnosis of Chronic Hepatitis B (2012). Only the patients diagnosed without any discrepancy by the three individual physicians were kept. Finally, 20 ADHS, 24 SDLD and, 20 BSVO patients were enrolled in the study. The patient population included 41 males (age: 23-77y, median 45y) and 23 females (age: 19-66y, median 50y) Chinese people of Han nationality. Their basic physiological parameters were referred to as Table S1. Informed consent was acquired from each patient. The study was approved by the Ethic Committee of Dalian Sixth People's Hospital (2016-004-002).

2.2 Clinical laboratory tests

Fasting blood samples were collected using ethylenediaminetetraacetic acid disodium salt as an anticoagulant. Plasma was acquired by centrifuging the whole blood samples at 5000 ×g for 3min. The plasma was stored at -80°C until further procession. The included clinical laboratory parameters included those of liver functions, kidney functions, blood cells count, ions, liver steatosis and so on (Table S1). Blood urea nitrogen, uric acid, cystatin, total protein, albumin, globulin, prealbumin, alanine transaminase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, lactic dehydrogenase, cholinesterase, monoamine oxidase, leucine aminopeptidase, 5'-nucleotidase, adenosine deaminase, alpha-L-fucosidase, creatine kinase, bile acid, cholyglycine, total bilirubin, direct bilirubin, and glucose were determined by SIEMENS ADVIA 2400 chemistry system (Tarrytown, NY). The utilized reagents were purchased from Ruiyuan (Ningbo, China), Daqian (Anhui, China) and Zhongyuan (Chongqing, China) and used according to the relevant instructions. The glomerular filtration rate was calculated using the formula described in the literature [16]. Blood potassium, sodium, chlorine, and carbon dioxide were measured by using VITROS 5.1 FS chemistry system (Ortho-Clinical

Diagnostics, Rochester, NY) with the kits provided by Ortho-Clinical Diagnostics. Blood cell parameters were determined by UniCel DxH 800 cellular analysis system (Beckman Coulter, Pompano Beach, FL) with the kits produced by Beckman Coulter. Blood prothrombin time, prothrombin activity, fibrinogen, active partial prothrombin time and thrombin time were determined by CA-1500 automated blood coagulation analyzer (Sysmex, Kobe, Japan). The reagents were purchased from Taiyang (Shanghai, China). $\beta2$-microglobulin, alpha fetal protein, carbohydrate antigen 199, and carcinoembryonic antigen were determined by Cobas e411 system (Roche. Mannheim, Germany) using the kits provided by the same company. Type IV collagen, laminin, procollagen III N-terminal peptide, and hyaluronic acid were quantified by HG-1000 time-resolved fluorescence immunoassay analyzer (Huguo, Shanghai, China) with its unique kits.

2.3 Sample processing for metabolomics and lipidomics analysis

Targeted metabolomic absolute quantitation assay was performed by using Biocrates Absolute*IDQ*™ p400 HR kits covering 408 metabolites and lipids (BIOCRATES Life Sciences AG, Innsbruck, Austria). The detailed detected metabolites were referred to as Table S1. Metabolomics analysis was performed by following the manufacturer's instructions. In brief, every 10 µL aliquots of internal standard mixture solution and 10 µL plasma were added into a 96-well filter plate well. Each sample plate was dried

for 30 min by nitrogen gas flow. Subsequently, every 50 µL aliquots of 5% phenylisothiocynate derivatization solution constructed in ethanol, water and pyridine mixture (1:1:1, v/v) were pipetted into each well. The plates were incubated at ambient temperature for 20 min followed by nitrogen drying for 60 min to finish derivatization. For each well, 300 µL of methanol containing 5 mM ammonium acetate was added to extract metabolites and lipids. Finally, the extraction solution was collected into a new 96-well capture plate under positive nitrogen pressure. For polar metabolite metabolomics analysis, every 150 µL of the extract was transferred into another empty 96-well plate and diluted with 150 µL of ultrapure water. For lipidomic analysis, every 250 µL of the mobile phase was added to each capture plate well.

2.4 Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) metabolomics analysis

The polar metabolites were analyzed using Thermo Scientific Ultimate 3000 ultraperformance liquid chromatography (UPLC) coupled with a Thermo Scientific Q Exactive Plus quadrupole-Orbitrap high-resolution mass spectrometry (MS) (San Jose, CA) operated under positive electrospray ionization mode. Every 5 µL of the diluted extract was injected into the UPLC system and separated by a Biocrates proprietary C_{18} column. The mobile phase A and B were 0.2% formic acid (v/v) water and 0.2% formic acid (v/v) acetonitrile solution, respectively. Full scan data with m/z range of 55~800

were acquired under 70,000 full width at half maximum (FWHM) resolution for polar metabolites quantification. The same UPLC-MS system was employed for lipidomic flow injection analysis (FIA) with each sample injection volume of 20 μL. Full scan with 70,000 FWHM resolution for each injection was segmented as m/z 150~170, 170~200, 200~240, 240~256, 390~520, 520~634, 634~730, 730~931 m/z, and 256~280, 280~305, 305~335, 335~363, 363~390, 390~415, 415~445 and 445~570, respectively. The LC-MS profiling data for polar metabolites were processed by using XCalibur Quan Brower software (Thermo Scientific). 40 amino acids, their derivates biogenic amines and 1 sugar (hexose, the sum of all six-carbon carbohydrates) were quantified by using the internal calibration method. The acquired FIA lipidomic data were directly imported into MetIDQ (BIOCRATES Life Sciences AG, Innsbruck, Austria) for lipid m/z extraction and quantitation (μmol/L).

2.5 Statistical analysis

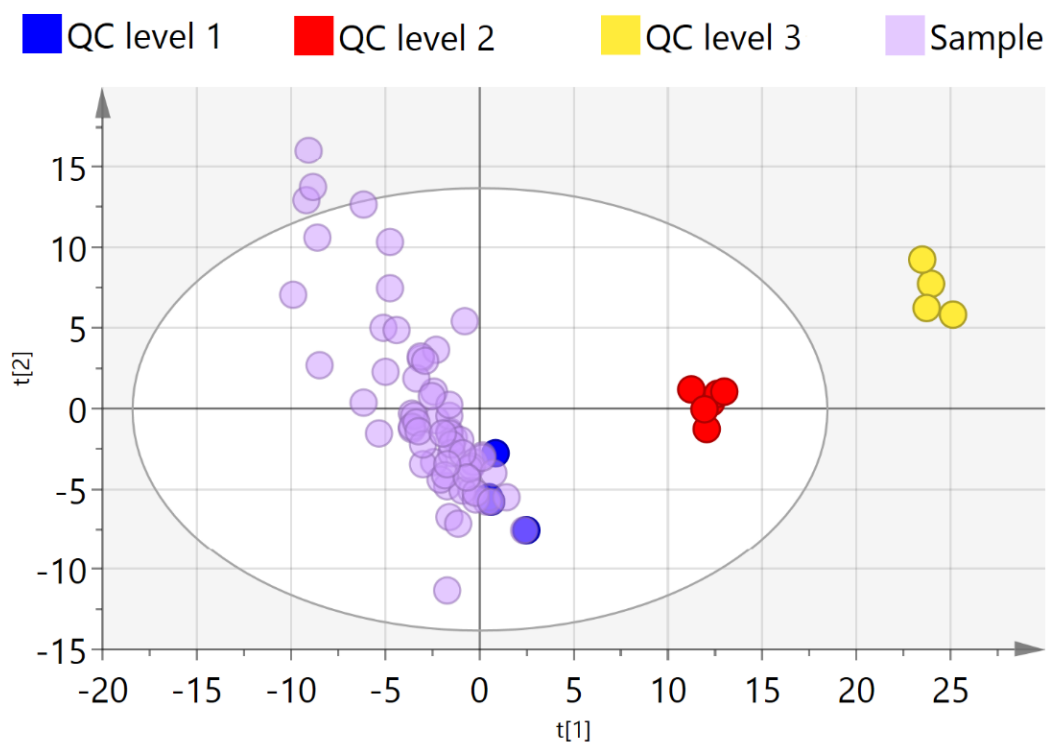To evaluate the metabolomics data quality, a principal component analysis (PCA) was conducted by using SIMCA-P v14.0 (Umetrics AB, Umeå, Sweden). For syndrome differentiation, the collected metabolomics and clinical laboratory data were processed using the algorithm of orthogonal partial least squares-discriminant analysis (OPLS-DA) as proposed and variables with variable importance for the projection (VIP) values >1 were deemed as meaningful [17]. For parameter comparison between every two syndromes, a *t-test* analysis was conducted by using Minitab V.17 (State College, PA).

3. Results

3.1 Metabolomics analysis reproducibility

To evaluate the analysis reproducibility, the lower, medium and higher concentrations quality control (QC) samples were randomly inserted into the real sample analysis queue and analyzed for 4, 6 and 4 replicate individually. PCA indicated that the three levels of QC samples were clustered well individually in the score plot (Fig. 1). The real samples tended to scatter in a large area. Distribution of the real samples was close to the lower level QCs samples, implying that the target metabolites were not very abundant in the patients' plasma.
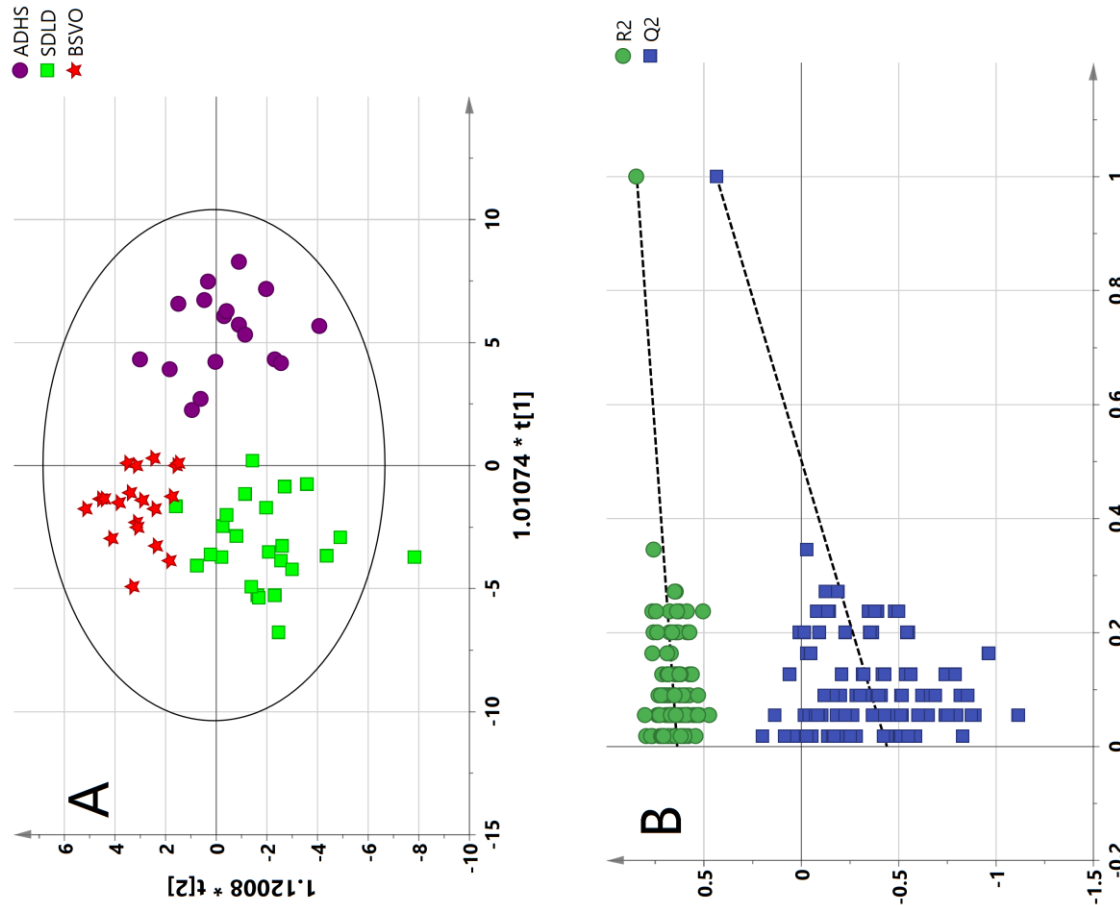
Figure 1 Method evaluation of the metabolomics analysis. Principal component analysis of the QCs and the real samples data indicated that every individual level of QCs clustered well, but the real samples scattered in a large range.

3.2 Syndrome differentiation

To pursue a better separation amid the three syndromes, routine clinical laboratory parameters were included in addition to the metabolomics data. An OPLS-DA indicated that the three syndrome groups could be separated acceptably (Fig. 2A). Permutations test based on 100 iterations indicated no over-fitting occurred in the DA model (Fig. 2B)[18].
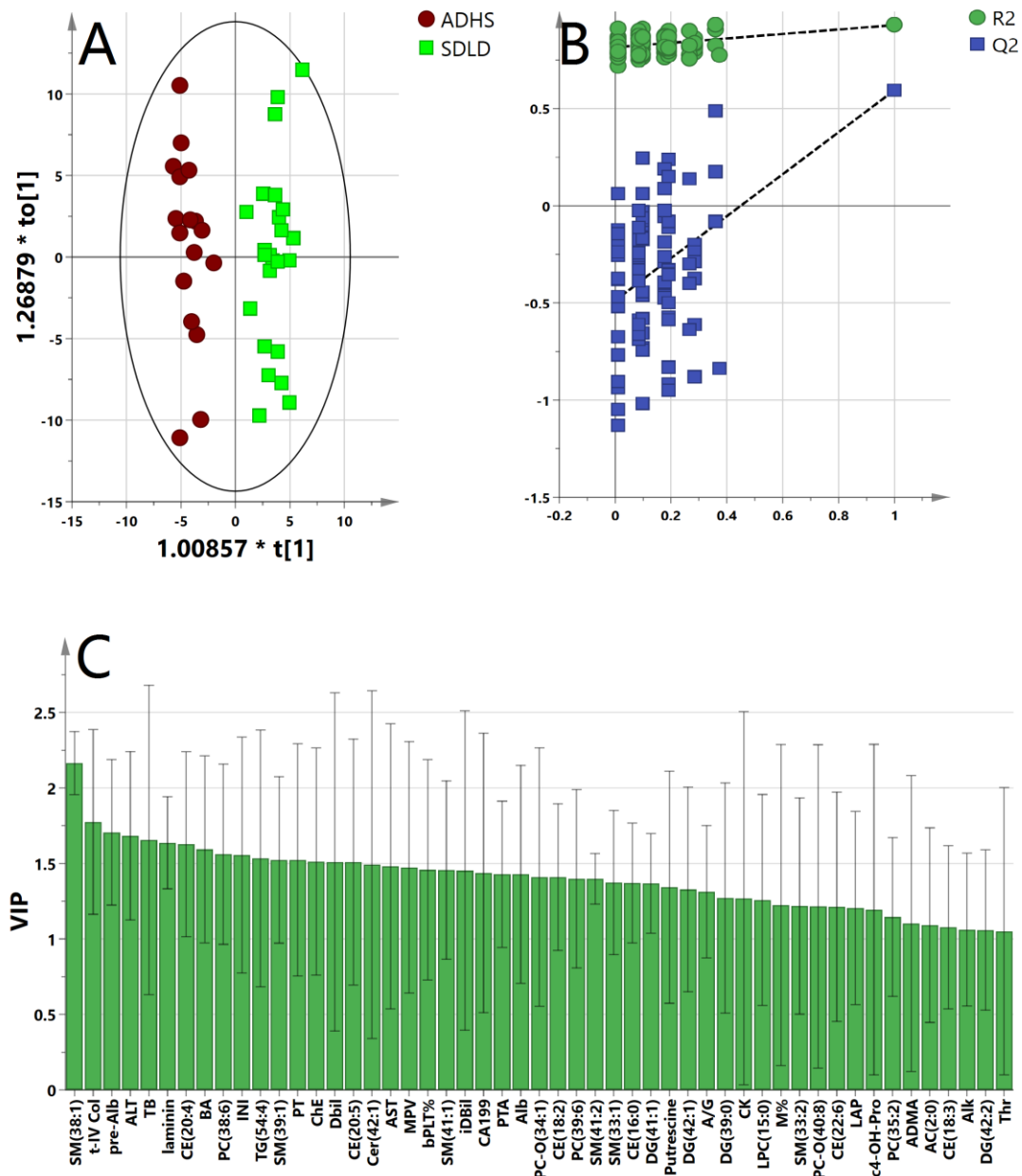
Figure 2 Differentiation of the three syndromes by orthogonal partial least squares-discriminant analysis. (A) Score plot of the three syndromes indicated the good separation amid them. (B) Permutations test based on 100 iterations indicated no over-fitting occurred in the DA model.

To explore the differential parameters amid the studied syndromes, every two syndromes data were subjected to OPLS-DA individually. 87 parameters contributed to the separation between ADHS and SDLD according to the parameters' VIP values (Fig. 3) [18]. 51 parameters showed statistical significance between the two syndromes (t-test, $p<0.05$, Fig. 3C). 75 parameters contributed to the separation of ADHS and BSVO. 42 parameters were of different concentrat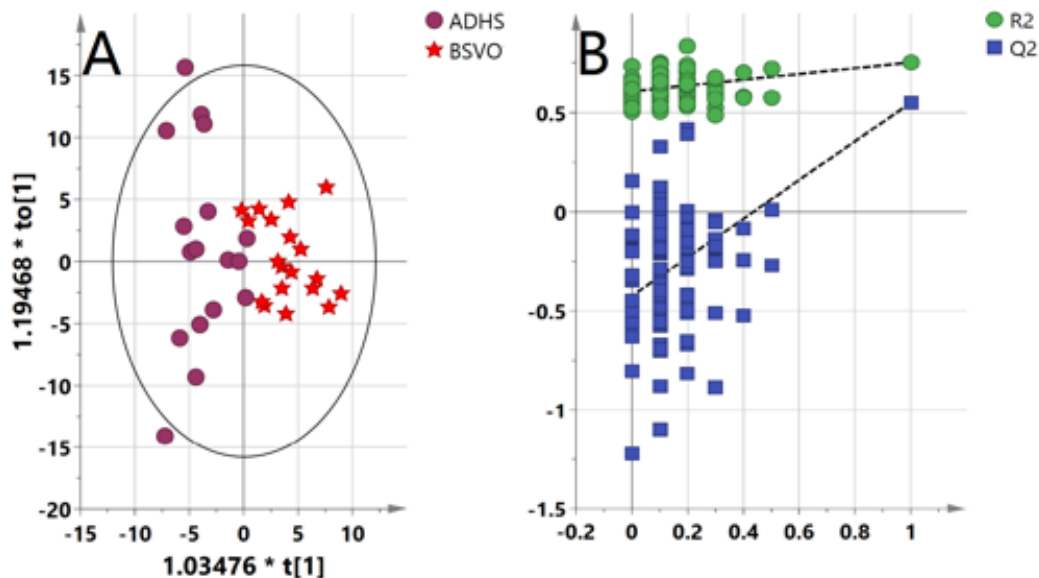ions between the two syndromes (t-test, $p<0.05$, Fig. 4). 50 parameters played key roles in separating SDLD from BSVO and only 16 of them were statistically different between the two syndromes (t-test, $p<0.05$, Fig. 5). Of note, there was no shared parameter showing a statistical difference between every two syndromes (Fig. 6A). This implied that the more complex the syndromes, the less the objective differential parameters. TCM clinical practice had demonstrated that complex syndromes tend to share more similarities and are indeed difficult to differentially diagnose clinically [19-21].
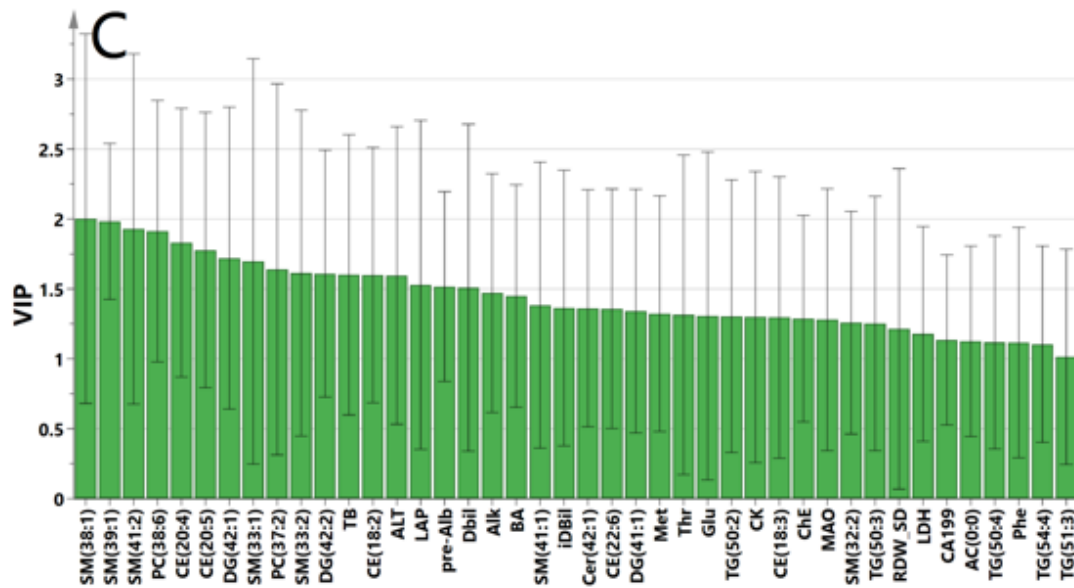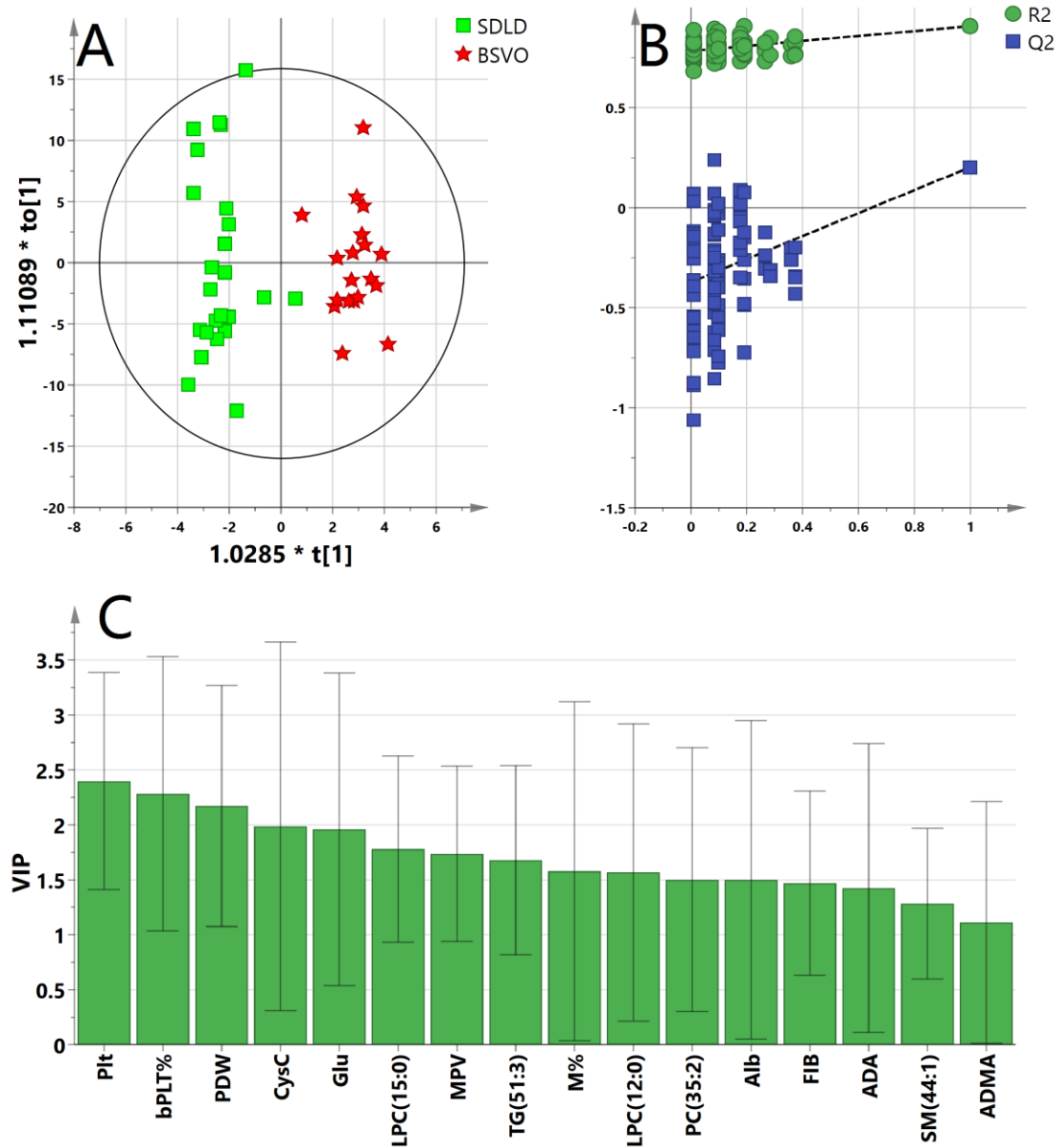
Figure 3 Differentiation of ADHS and SDLD by orthogonal partial least squares-discriminant analysis. (A) Score plot indicated a good separation of the two syndromes. (B) Permutations test based on 100 iterations indicated no over-fitting occurred in the DA model. (C) Differential variables between ADHS and SDLD. All the parameters were of variable importance for the projection values>1 in OPLS-DA and p<0.05 of t-test. SM(38:1): sphingomyelin(38:1); t-IV Col: type IV collagen; pre-Alb: prealbumin; ALT: alanine transaminase; TB: total bilirubin; CE(20:4): Cholesterol ester(20:4); BA: bile acid; PC(38:6): phosphatidylcholine(38:6); INI: international normalized ration; TG(54:4): triglyceride(54:4); SM(39:1): sphingomyelin(39:1); PT: prothrombin time; ChE: cholinesterase; Dbil: direct bilirubin; CE(20:5): cholesterol ester(20:5); Cer(42:1):

ceramide(42:1); AST: aspartate aminotransferase; MPV: mean platelet volume; bPLT%: big platelet percentage; SM(41:1): sphingomyelin(41:1); iDBil: indirect bilirubin; CA199: carbohydrate antigen 199; PTA: prothrombin activity; Alb: albumin; PC-O(34:1): phosphatidylcholine-O(34:1); CE(18:2): cholesterol ester(18:2); PC(39:6): phosphatidylcholine(39:6); SM(41:2) sphingomyelin(41:2); SM(33:1): sphingomyelin(33:1); CE(16:0): cholesterol ester(16:0); DG(41:1): diglyceride(41:1); DG(42:1): diglyceride(42:1); A/G: albumin/globulin; DG(39:0): diglyceride(39:0); CK: creatine kinase; LPC(15:0): lysophosphatidylcholine(15:0); M%: monocyte percentage; SM(33:2): sphingomyelin(33:2); PC-O(40:8): phosphatidylcholine-O(40:8); CE(22:6): cholesterol ester(22:6); LAP: leucine aminopeptidase; c4-OH-Pro: cis4-OH-proline; PC(35:2): phosphatidylcholine(35:2); ADMA: asymmetric dimethylarginine; AC(2:0):acylcarnitine(2:0); CE(18:3): cholesterol ester(18:3); Alk: alkaline phosphatase; DG(42:2): diglyceride(44:2) and Thr: threonine.
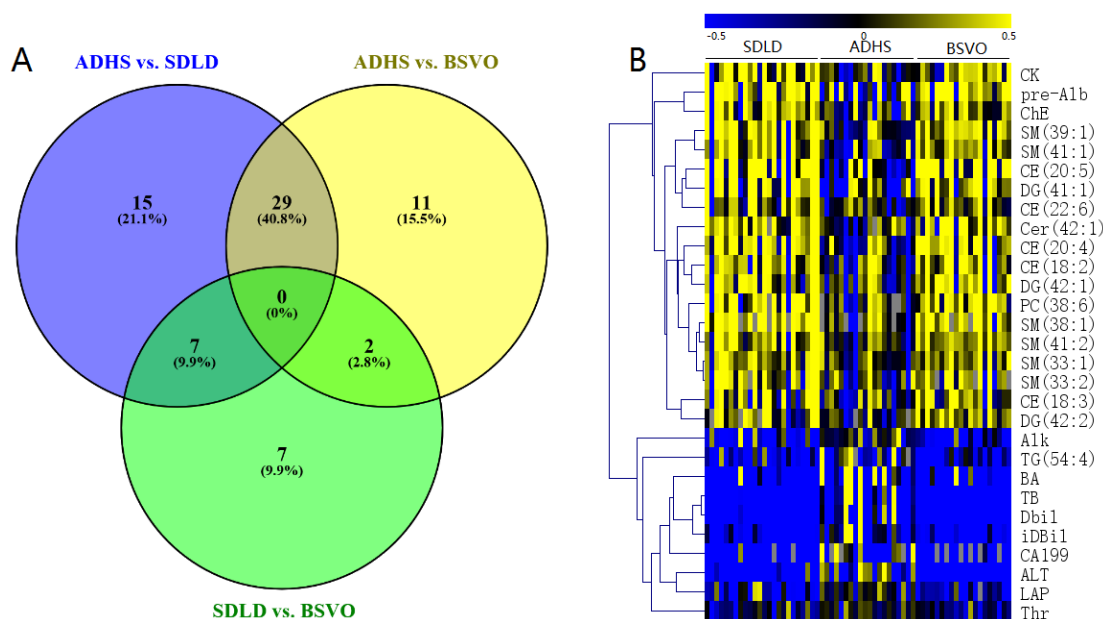
Figure 4 Differentiation of ADHS and BSVO by orthogonal partial least squares-discriminant analysis. (A) Score plot indicated a good separation of the two syndromes. (B) Permutations test based on 100 iterations indicated no over-fitting occurred in the DA model. (C) Differential variables between ADHS and BSVO. The selection criteria were the same as described in Fig.2C. PC(37:2): phosphatidylcholine(37:2); Met: methionine; Glu: glutamate; TG(50:2): triglyceride(50:2); MAO: monoamine oxidase; SM(32:2): sphingomyelin(32:2); TG(50:3) triglyceride(50:3); RDW_SD: CV of red blood cell volume distribution width; LDH: lactic dehydrogenase; AC(0:0): acylcarnitine(0:0); TG(50:4): triglyceride(50:4); Phe: phenylalanine and TG(51:3): triglyceride(51:3).

Figure 5 Differentiation of SDLD and BSVO by orthogonal partial least squares-discriminant analysis. (A) Score plot indicated a good separation of the two syndromes. (B) Permutations test based on 100 iterations indicated no over-fitting occurred in the DA model. (C) Differential variables between SDLD and BSVO. The selection criteria were the same as described in Fig.2C. Plt：platelet; PDW: platelet volume distribution width; CysC: cystatin C; LPC(12:0): lysophosphatidylcholine(12:0); FIB: fibrinogen; ADA: adenosine deaminase; SM(44:1): sphingomyelin(44:1).

Figure 6 Differential parameters of the comparisons of the three syndromes. (A) Numbers of differential parameters shared by each comparison. (B) The plasm concentration fold changes of the differential parameters from comparison between ADHS and the other two syndromes. All the concentrations were normalized against the means of corresponding variable concentrations of ADHS. The fold changes were presented after natural logarithm conversion.

It was also found that decreased glutamate (Glu) and triglyceride (TG) (51:3) were the most important two differential parameters between BSVO and the other two syndromes. Increased lysophosphatidylcholine(15:0) LPC15:0), phosphatidylcholine (PC)(35:2), mean plate volume(MPV) and albumin with decreased big platelet percentage (bPLT%), monocyte percentage (M%) and asymmetric dimethylarginine (ADMA) were statistically significant between SDLD and the others syndromes. There were 29 different parameters between ADHS and the other syndromes. Most of them were sphingomyelins (SMs) and cholesterol esters (CEs) (Fig. 6B).

4. Discussion

Traditionally, the syndrome differentiation in TCM is based on the expertise of the physicians. Amid chronic HBV infection patients ADHS, SDLD, and BSVO are three commonly encountered syndromes in TCM clinical practice. According to the metabolomics and the routine clinical laboratory parameters, it was apparent that the three syndromes were distinct (Fig. 2). From Fig. 5 and 6A, the difference between

SDLD and BSVO seemed trivial. Only 16 parameters were different between them. This could also be proved by both the clinical manifestations of the typical patients and the acknowledged TCM theory. To be frank, sometimes, even an experienced TCM physician also feels it is a challenge to accurately differentiate SDLD and BSVO [19-21].

Compared to the other two syndromes, the lowered metabolites in ADHS were mainly SMs, CEs and diglycerides (DGs) (Fig. 6B). SMs are synthesized from ceramide (Cer) by sphingomyelin synthases (SMSs) [22]. Interestingly, reactions catalyzed by SMSs can yield both SMs and DGs simultaneously [22]. Three SMSs homologs (SMS1, SMS2, and SMS related proteins) had been identified to date. The SMS related proteins have no enzymatic activity. SMS1 and SMS2 are functionally similar [23]. Up to now, the extensive functional studies about SMs are seldom, but suppression of SMSs results in decreased SMs. Using SMS1 and SMS2 knockout mice, some phenotype changes were reported and many of the features were similar to what had been typically described in the ADHS. For example, SMS1 knockout mice exhibited decreasing body weight and loss of adipose tissue [24]. Similar phenomena can result from chronic loose stools, which is usually found in ADHS patients. The loss of adipose tissue might explain why the lower content of CE in ADHS (Fig. 6B). SMS2 knockout mice showed not only lowered plasma[25] and brain SMs[26] but also decreased liver steatosis [27]. In clinical TCM practice, liver steatosis was seldom found in ADHS but was frequently encountered in BSVO and SDLD. Compared to ADHS, the other two syndromes were characterized with much higher levels of some typical liver functions' parameters (Fig. 6B). Some of them even slightly exceed the normal ranges (Table S1). Empirically, TCM physicians believe that SDLD and BSVO are the advanced stages of ADHS. From Fig. 6, it was also clear that TB elevated in ADHS but not in the other two syndromes. In TCM theory, long time dampness-heat will cause "bile leakage", which is called jaundice in the Western Medicine theory. The appearance of jaundice usually implies liver injury.

SDLD is often accompanied by abnormal spleen functions. In this study, the syndrome was linked to some platelet parameters, such as the lower bPLT% and higher MPV. The conception of "spleen" in TCM theory is not equal to that of Western Medicine. The exact reasons for the changed platelet parameters were elusive. PCs and LPCs were elevated in SDLD. They are involved in varied kinds of inflammatory reactions. Recently, platelets were found to play key roles in modulating PCs and LPCs -involved inflammation [28]. The higher mean platelet volume was found in maternal blood and could be used to predict the early onset of sepsis of the infants[29]. Another study demonstrated that elevated mean platelet volume was an indicator of fibrosis and was positively correlated to the fibrosis degree in chronic hepatitis C virus infected patients[30]. Intrahepatic

cholestasis of pregnancy could exhibit higher MPV[31], and intrahepatic cholestasis can be frequently found in SDLD. These clues might imply that SDLD was affected by certain biological or pathological functions of platelets to some extent.

5. Conclusion

In this study, by combined using metabolomics and clinical laboratory data, the typical 3 syndromes of HBV infection patients were compared. The ADHS phenotype was deduced to be closely related to SMs metabolism. The SDLD phenotype might be affected by platelet functions. BSVO was distinct to ADHS but was more similar to SDLD phenotypically given the employed data. The differential parameters amid the studied syndromes might provide valuable help in TCM practice. As had been mentioned above, to define a TCM syndrome is solely dependent on the physicians' experience. Thus, for 3 TCM physicians, to agree with each other without any discrepancy is difficult. This fact resulted in the number of qualified patients with definite syndrome was limited. It was highly warranted that the discovered differential parameters were verified in a large patient population. Of note, most of the differential parameters were small molecular metabolites. This also proved that metabolomics analysis could help the old TCM to progress to modernization.

Acknowledgment

6. References

1. Ogawa E, Furusyo N, Nguyen MH, Tenofovir alafenamide in the treatment of chronic hepatitis B: design, development, and place in therapy. Drug Des Devel Ther 2017; 11:3197-3204.DOI:10.2147/DDDT.S126742

2. Li F, Shao Q, Ji D, Li B, Chen G, Genetic association between CD44 polymorphisms and chronic hepatitis B virus infection in a Chinese Han population. Int J Clin Exp Pathol 2015; 8(9):11675-11679.

3. Chen T, Qian G, Fan C, Sun Y, Wang J, Lu P *et al*, Qidong hepatitis B virus infection cohort: a 25-year prospective study in high risk area of primary liver cancer. Hepatoma Res 2018; 410. DOI:20517/2394-5079.2017.50

4. Stanaway JD, Flaxman AD, Naghavi M, Fitzmaurice C, Vos T, Abubakar I *et al*, The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. Lancet 2016; 388(10049):1081-1088. DOI:10.1016/S0140-6736(16)30579-7

5. Qi FH, Wang ZX, Cai PP, Zhao L, Gao JJ, Kokudo N *et al*, Traditional Chinese medicine and related active compounds: a review of their role on hepatitis B virus infection. Drug Discov Ther 2013; 7(6):212-224.

6. Lau DT, Bleibel W, Current status of antiviral therapy for hepatitis B. Therap Adv Gastroenterol 2008; 1(1):61-75. DOI:10.1177/1756283X08093944

7. Hadziyannis SJ, Update on Hepatitis B Virus Infection: Focus on Treatment. J Clin Transl Hepatol 2014; 2(4):285-291. DOI:10.14218/JCTH.2014.00026

8. Dobos GJ, Tan L, Cohen MH, McIntyre M, Bauer R, Li X *et al*, Are national quality standards for traditional Chinese herbal medicine sufficient? Current governmental regulations for traditional Chinese herbal medicine in certain Western countries and China as the Eastern origin country. Complement Ther Med 2005; 13(3):183-190. DOI:10.1016/j.ctim.2005.06.004

9. Wang L, Zhang L, Feng X, Xing L, Zhang W, Jiang K *et al*, The Functional Difference of Dendritic Cells in HBeAg Negative Chronic Hepatitis B Patients with Three Different Spleen Deficiency Syndromes and the Therapeutic Evaluation of Chinese Medicine Intervention Based on Syndrome Differentiation. Evid Based Complement Alternat Med 2014; 2014:802402. DOI:10.1155/2014/802402

10. Zhao Y, Peng JH, Li XM, Fu QL, Cui T, Li Q *et al*, [Diagnostic value of clinical indices in syndrome differentiation of chronic hepatitis B: an exploration based on receiver operating characteristic curves and stepwise discriminant analysis]. Zhong Xi Yi Jie He Xue Bao 2012;
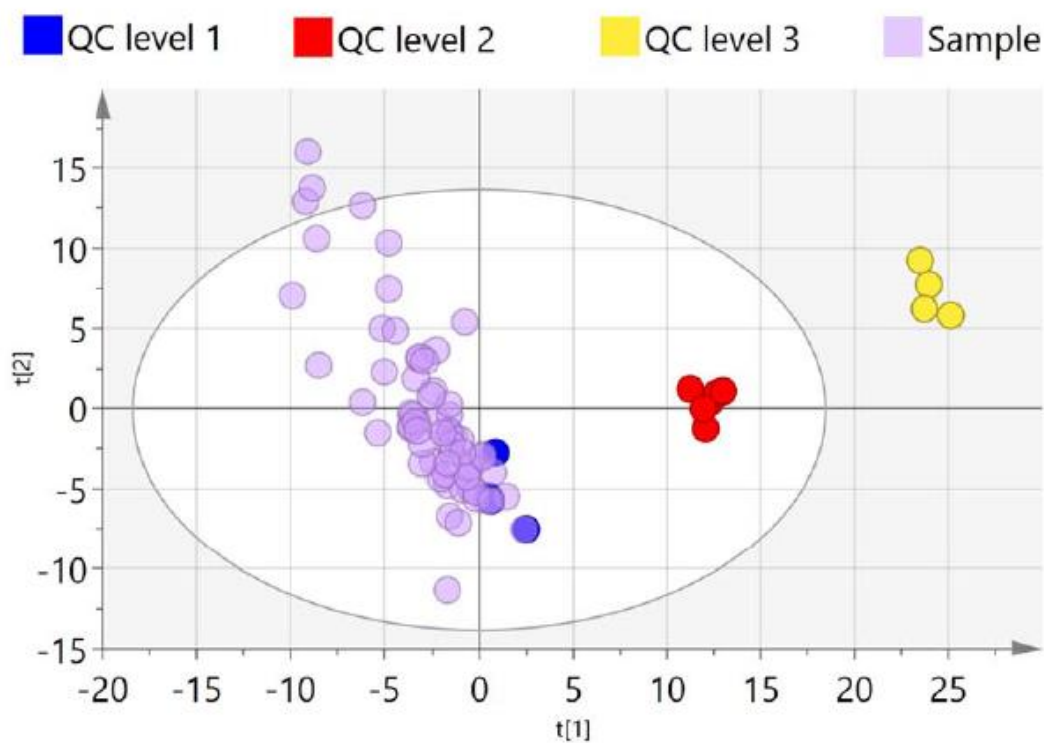
10(12):1382-1387.
DOI:10.3736/jcim20121208

11. Lang QB, Zhai DX, Huang F, Chen JG, Zhang YH, Liu Q *et al*, [Investigation on traditional Chinese medicine syndrome distribution of 4 618 hepatitis B virus infection subjects in Qidong of Jiangsu Province, China]. Zhong Xi Yi Jie He Xue Bao 2012; 10(5):525-531.

12. Hollywood KA, Schmidt K, Takano E, Breitling R, Metabolomics tools for the synthetic biology of natural products. Curr Opin Biotechnol 2018; 54:114-120. DOI:10.1016/j.copbio.2018.02.015

13. van der Greef J, van Wietmarschen H, Schroen J, Wang M, Hankemeier T, Xu G, Systems biology-based diagnostic principles as pillars of the bridge between Chinese and Western medicine. Planta Med 2010; 76(17):2036-2047. DOI:10.1055/s-0030-1250450

14. Siristatidis CS, Sertedaki E, Vaidakis D, Varounis C, Trivella M, Metabolomics for improving pregnancy outcomes in women undergoing assisted reproductive technologies. Cochrane Database Syst Rev 2018; 3:CD011872. DOI:10.1002/14651858.CD011872.pub3

15. Ghanbari R, Sumner S, Using Metabolomics to Investigate Biomarkers of Drug Addiction. Trends Mol Med 2018; 24(2):197-205.

DOI:10.1016/j.molmed.2017.12.005

16. Antlanger M, Dust T, Reiter T, Bohm A, Lamm WW, Gornicec M *et al*, Impact of renal impairment on outcomes after autologous stem cell transplantation in multiple myeloma: a multi-center, retrospective cohort study. BMC Cancer 2018; 18(1):1008. DOI:10.1186/s12885-018-4926-0

17. Pasikanti KK, Esuvaranathan K, Hong Y, Ho PC, Mahendran R, Raman Nee Mani L *et al*, Urinary metabotyping of bladder cancer using two-dimensional gas chromatography time-of-flight mass spectrometry. J Proteome Res 2013; 12(9):3865-3873. DOI:10.1021/pr4000448

18. Huang Z, Lin L, Gao Y, Chen Y, Yan X, Xing J *et al*, Bladder cancer determination via two urinary metabolites: a biomarker pattern approach. Mol Cell Proteomics 2011; 10(10):M111 007922. DOI:10.1074/mcp.M111.007922

19. Jiang M, Lu C, Zhang C, Yang J, Tan Y, Lu A *et al*, Syndrome differentiation in modern research of traditional Chinese medicine. J Ethnopharmacol 2012; 140(3):634-642.
DOI:10.1016/j.jep.2012.01.033

20. Gu Z, Qi X, Zhai X, Lang Q, Lu J, Ma C *et al*, Study on TCM Syndrome Differentiation of Primary Liver Cancer Based on the Analysis of Latent Structural Model. Evid Based Complement

Alternat Med 2015; 2015:761565. DOI:10.1155/2015/761565

21. Jiansheng L, Haifeng W, Suyun L, Hailong Z, Xueqing Y, Xiaoyun Z *et al*, Effect of sequential treatment with TCM syndrome differentiation on acute exacerbation of chronic obstructive pulmonary disease and AECOPD risk window. Complement Ther Med 2016; 29:109-115. DOI:10.1016/j.ctim.2016.09.009

22. Ding T, Li Z, Hailemariam T, Mukherjee S, Maxfield FR, Wu MP *et al*, SMS overexpression and knockdown: impact on cellular sphingomyelin and diacylglycerol metabolism, and cell apoptosis. J Lipid Res 2008; 49(2):376-385. DOI:10.1194/jlr.M700401-JLR200

23. Shakor AB, Taniguchi M, Kitatani K, Hashimoto M, Asano S, Hayashi A *et al*, Sphingomyelin synthase 1-generated sphingomyelin plays an important role in transferrin trafficking and cell proliferation. J Biol Chem 2011; 286(41):36053-36062.
DOI:10.1074/jbc.M111.228593

24. Yano M, Watanabe K, Yamamoto T, Ikeda K, Senokuchi T, Lu M *et al*, Mitochondrial dysfunction and increased reactive oxygen species impair insulin secretion in sphingomyelin synthase 1-null mice. J Biol Chem 2011; 286(5):3992-4002.
DOI:10.1074/jbc.M110.179176

25. Liu J, Huan C, Chakraborty M, Zhang H, Lu D, Kuo MS *et al*, Macrophage sphingomyelin synthase 2 deficiency decreases atherosclerosis in mice. Circ Res 2009; 105(3):295-303. DOI:10.1161/CIRCRESAHA.109.194613

26. Zhang Y, Dong J, Zhu X, Wang W, Yang Q, The effect of sphingomyelin synthase 2 (SMS2) deficiency on the expression of drug transporters in mouse brain. Biochem Pharmacol 2011; 82(3):287-294.
DOI:10.1016/j.bcp.2011.04.009

27. Mitsutake S, Zama K, Yokota H, Yoshida T, Tanaka M, Mitsui M *et al*, Dynamic modification of sphingomyelin in lipid microdomains controls development of obesity, fatty liver, and type 2 diabetes. J Biol Chem 2011; 286(32):28544-28555. DOI:10.1074/jbc.M111.255646

28. Marathe GK, Pandit C, Lakshmikanth CL, Chaithra VH, Jacob SP, D'Souza CJ, To hydrolyze or not to hydrolyze: the dilemma of platelet-activating factor acetylhydrolase. J Lipid Res 2014; 55(9):1847-1854. DOI:10.1194/jlr.R045492

29. Cekmez Y, Dizdar Gulecoglu M, Ozcan C, Karadeniz L, Kiran G, The utility of maternal mean platelet volume levels for early onset neonatal sepsis prediction of term infants. Ginekol Pol 2017; 88(6):312-314.
DOI:10.5603/GP.a2017.0058

30. Gozdas HT, Ince N, Elevated mean platelet volume to platelet ratio predicts advanced fibrosis in

chronic hepatitis C. Eur J Gastroenterol Hepatol 2020; 32(4):524-527. DOI:10.1097/MEG.0000000000001599

31.   Cho SY, Lee HJ, Park TS, Mean platelet volume in patients with increased gamma-glutamyl transferase. Platelets 2015; 26(3):283-284. DOI:10.3109/09537104.2014.881467

Supplementary material description

Table S1 The collected clinical parameters and the metabolites detected in metabolomics analysis [available in a separate file upon request]

Figure S1: method evaluation of the metabolomics analysis. Principle component analysis of the QCs and real samples data indicated that every individuals level of QCs clustered well,, but the real samples scattered in large range.