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Metabolomics of Alcoholic Liver Disease: A Clinical Discovery Study

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Abstract:

Alcoholic liver disease (ALD) is associated with poor health and disease dysfunction in worldwide. Unfortunately, current biomarkers including PCIII, IV-C, LN, HA levels, are expensive and lack sensitivity in ALD detection. Because they are either invasive, time-consuming or expensive, and ALD is usually diagnosed at late stages, for which there are no effective therapies. Thus, biomarkers for early detection of ALD are urgently needed. Thankfully, metabolomics is a powerful technology that allows the assessment of global low-molecular-weight metabolites in a biological system and which shows great potential in biomarker discovery. Analysis of the key metabolites in body fluids has become an important part of improving the diagnosis, prognosis, and therapy of diseases. Urine biomarkers may be a more attractive option, but none can currently detect ALD disease with the required accuracy. Herein, we describe our metabolomics approach to detecting ALD disease in a group of 206 patients. A total of 6 urinary differential metabolites were identified and contributed to ALD progress, and more important, we discovered 3 of them with over 95% accuracy. The biomarker panel may be sensitive to early diagnosis, prognosis and therapy of ALD disease.

Keywords:

Alcoholic liver disease; metabolomics; metabolites; biomarkers; urine

Introduction

Alcohol misuse is a major public health problem in the worldwide and accounts for elevated social and economic costs [1]. Alcoholic liver disease (ALD), which is a liver disease because of alcohol consumption, is a common complication of alcohol misuse. By 2050, an estimated 165 million individuals worldwide will have ALD, consuming an estimated $120 trillion in health care costs per year [2]. Unfortunately, current biomarkers (including PCIII, IV-C, LN, HA) for early disease are expensive and lack sensitivity in ALD detection [3-6]. The high cost associated with these technologies is a significant barrier to widespread use in clinical practice. They have only been shown to be effective in confirming the diagnosing of the diseases after the ALD symptoms have surfaced [7]. The challenge is there currently exists no way to identify which people are at risk of developing ALD. Thus, it is important to develop more effective methods for noninvasive early diagnosis of this disease process, also there is an urgent need for biomarkers to diagnose ALD. Fortunately, metabolomics technology has been used to explore the particular metabolites, potentially diagnostic biomarkers for deep understanding the essence of diseases [8-10].

At the end of the 20th century, genomics wrote out the ‘script of life’; proteomics decoded the script; and metabolomics came into bloom [11]. Metabolomics is the endpoints of genotype functions and biochemical phenotype in body, are linked closely to functions alteration, and incorporates a ‘top-down’ strategy to reflect the terminal symptoms of a whole system and facilitates biomarker discovery [12]. Urine is an ideal bio-medium for disease study because it is readily available, easily obtained and less complex than other body fluids [13,14]. Ease of collection allows for serial sampling to monitor disease and therapeutic response. Numerous researchers have revealed the potential role of plasma metabolomics in searching for biomarkers predictive of therapeutic responses [15]. Despite this expansion, there is no report of integrative study on urine metabolomics of ALD. In an attempt to address this issue, we used urine metabolomics approach analyzed detecting ALD disease in a group of 206. This study was performed by high-throughput UPLC/ESI-Q/TOF-MS metabolomics combined with pattern recognition analysis multiplatform which were used to select the marker metabolites.

2. Materials and Methods

2.1 Reagents

Acetonitrile and methanol were purchased from Fisher Scientific Corporation (New Jersey, USA). High purity formic acid (99%) was purchased from Honeywell Company (Morristown, New Jersey, USA). water was produced by a Milli-Q Ultra-pure water system (Millipore, Billerica, USA); leucine enkephalin was purchased from Sigma-Aldrich. All other reagents were HPLC grade.

2.2 Human subjects

The clinical specimens, including 206 human urine samples from ALD patients and corresponding 101 normal urine samples, were obtained from the First Affiliated Hospital, Heilongjiang University of Chinese Medicine. The

diagnoses of these samples were verified by pathologists. All of the samples were obtained with the informed consent of the patients and approved by the Ethics Committee of Heilongjiang University of Chinese Medicine (HUCM-2013-3152) and was conducted according to the Declaration of Helsinki.

2.3 Preparation of urine samples

Briefly, the urine samples were thawed on ice and vortexed at 10,000 rpm for 10 minutes at 4◦C to remove fine particulates. The supernatant was transferred to a glass vial and then filtered through a 0.22 μm syringe filter, 3 μL of the supernatant were injected into liquid chromatography-electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS) analysis.

2.4 LC-ESI-QTOF-MS analysis

All samples were analysed using an LC system (Waters Corp., Milford, USA), equipped with an ACQUITY BEH C_{18} chromatography column (100 mm×2.1mm i.d., 1.7μm, Waters Corporation, Milford, USA). The column temperature was maintained at 45◦C, and then gradient mobile phase was composed of phase A (water with 0.1% formic acid) and phase B (acetonitrile containing 0.1% formic acid). The gradient for the urine sample was as follows: 0–5 min, 1–55% B; 5–9.5 min, 55–50% B; 9.5–9.6 min, 50–1% B; 9.6–11 min, 1% B; 11–11.1 min, 1–99% B; 11.1–13 min, 99% B. The injection volume was 3 μL and the flow rate of the LC system was 0.4 mL/min. After every 10 sample injections, a pooled blank (quality control) sample was injected in order to ensure the stability and repeatability of the LC-MS system. All samples were maintained at 4 °C during the analysis.

Mass spectrometry was performed on a quadrupole time-of-flight (Q-TOF) instrument (Waters Corp., Milford, USA) operating in either negative (ESI^-) or positive (ESI^+) electrospray ionization mode with a capillary voltage of 3,200 V in positive mode and 2,800 V in negative mode and a sampling cone voltage of 30 V in both modes. The source temperature was set at 120 °C. The desolvation temperature was set to 350◦C, desolvation gas flow rate was set to 500 L/h, and cone gas flow was set at 50 L/h. Accurate mass was maintained by introduction of a lock-spray interface of leucine-enkephalin (556.2771 $[M+H]^+$ or 554.2615 $[M-H]^-$) at a concentration of 0.2 ng/mL in a flow rate of $100\mu l \cdot min^{-1}$. Data were acquired in centroid MS mode from 100 to 1 500 *m/z* mass range for TOF-MS scanning as single injection per sample, and the batch acquisition was repeated to check experimental reproducibility. For the metabolomics profiling experiments, pooled quality control samples (generated by taking an equal aliquot of all the samples included in the experiment) were run at the beginning of the sample queue for column conditioning and every ten injections thereafter to assess inconsistencies that are particularly evident in large batch acquisitions in terms of retention time drifts and variation in ion intensity over time.

2.5 Statistical analyses

All the LC-MS raw files were converted to EZinfo software (which is included in MarkerLynx Application Manager and can be applied directly) for compound statistics (principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA)), correlation analysis and compound validation. The combining VIP-plot

from the OPLS-DA was carried out to select distinct variables as potential markers. Metabolite identification was determined as follows: first, the possible fragment mechanism was searched by MassFragment™ manager (Waters Corp., Milford, USA) which was used to facilitate the MS fragment ion analysis process by way of chemically intelligent peak-matching algorithms; and then the tandem mass was carried out and structure information of metabolites were matched from HMDB and METLIN; finally, the chemical structures of the candidate metabolites were confirmed. The classification performance of the selected metabolites was assessed using area under the ROC curve (AUC). The ROC can be understood as a plot of the probability of classifying correctly the positive samples against the rate of incorrectly classifying true negative samples. The AUC measure of an ROC plot is a measure of predictive accuracy. All statistical analyses were performed using the Student's t-test. Differences with a P-value of 0.05 or less were considered significant.

3. Results

3.1 Urine metabolite profiles

In this study, a total of 206 human urine samples from ALD patients and corresponding 101 normal urine samples were collected and analyzed both by LC-ESI-QTOF-MS. **Fig. 1** presents the total ion current chromatograms of the control subjects and ALD patients in positive mode. BPI exhibited the ideal separation result under the optimized gradient procedure. For further analysis of the metabolic differences between the ALD and control group, all of the raw data from LC/MS ions were imported into the EZinfo 2.0 package.

3.2 Multivariate statistical analysis of metabolite profiles

Multivariate data analysis was performed using the score plot of PCA, and there is an obvious separation between the clustering of the ALD and control groups (see **Fig. 2 A and B**), suggests that biochemical perturbation significantly happened in ALD group. For further analysis of feature ions, the S-plot combined VIP- plot from the OPLS were to select variables as potential markers for distinguishing ALD patients from controls (**Fig. 2 C and D**). We generated VIP plots from the OPLS-DA with a threshold of 1.5 to identify the metabolites that significantly contribute to the clustering between groups. Six differentially expressed metabolites from ALD patients were distinguished from those of the controls ($p < 0.05$, VIP>1.5, 2 ions in the positive mode and 4 ions in negative mode). The VIP plot displayed 6 ions as differentiating metabolites according to their VIP values and considered as potential markers representing the metabolic characteristics (**Table 1**). According to the protocol detailed above, 6 endogenous metabolites were finally identified as markers and listed in **Table 1**. Overall, these metabolites displayed considerable differences between control and ALD samples, including sebacic acid, 3-hydroxytetradecanedioic acid, isocitric acid, suberic acid, isoamyl salicylate, 6-methylquinoline. These metabolites were unambiguously identified using tandem mass spectrometry. It was found that, among them, four compounds were upregulated and two compounds were downregulated.

3.3 Confirmation of clinical diagnosis for marker metabolites

We enrolled 206 ALD participants that met criteria (Supplementary Table 1), aged 30 and older and otherwise healthy, into this 2-year observational study. The validation samples were obtained from those clinically defined ALD subjects. Clinical estimation of markers with ROC analysis was determined in urine samples from ALD patients and 101 volunteers to evaluate the metabolite profile for diagnosing ALD. We examined the urine samples from the participants for untargeted metabolomic analysis. The samples were processed and analyzed using the same UPLC-MS technique as in the discovery phase. Metabolomic profiling yielded 6,200 positive-mode features and 4,700 negative-mode features. A notable finding of this targeted metabolomic analysis was the identification of a set of 6 metabolites, comprising sebacic acid, 3-hydroxytetradecanedioic acid, isocitric acid, suberic acid, isoamyl salicylate, 6-methylquinoline. Studies have shown decreased urine sebacic acid level and increased 3-hydroxy tetradecanedioic acid, isocitric acid metabolites in patients with ALD [16]. To detect the expression of a set of metabolites in human ALD urine, the LC-MS assay was performed on urine samples of ALD and normal urine. The targeted quantitative analysis of the validation set revealed the levels for the six-metabolite panel (**Fig. 3**) as were observed in the discovery samples. We used receiver operating characteristic (ROC) analysis to assess the performance of the classifier models for group classification. The ROC analysis revealed 3-hydroxytetradecanedioic acid, isocitric acid, sebacic acid to be potent discriminators of the between control and ALD groups (**Fig. 4**). For the ALD group classification, the initial identified metabolites 3-hydroxytetradecanedioic acid, isocitric acid, sebacic acid yielded a robust area under the curve (AUC) of 0.997 (**Fig.4A**), 0.993 (**Fig.4B**), 0.978 (**Fig.4C**) for ALD group classification. The predictive value of these biomarkers in preclinical patients is strong, suggesting that these markers may be useful for confirmation of clinical diagnosis in the near future.

Discussion

ALD is a major cause of alcohol-related morbidity and mortality [17]. Analysis of the key metabolites in body fluids has become an important part of improving the diagnosis, prognosis, and therapy of diseases [18]. Firstly, liver histology combined with biochemical results, we successfully established liver fibrosis in animal models. A panel of biomarkers to characterise disease could be useful for ALD diagnostics. In this paper, LC-MS combined with pattern recognition analysis approach were used to simplify and quicken the identification of the metabolites of ALD. LC-MS based metabolomics could be an advanced tool to help us find metabolites with regards to its capacity of processing large datasets, and classifying of sample groups, as well as its indiscriminative nature of metabolites [19,20]. By using our metabolomics platform, PCA revealed a significant separation between the ALD and control samples. OPLS model was built to find biomarkers of ALD and 6 statistically important variables with VIP>1.5 were defined, many are in various stages of progress at the ALD. We used the metabolomic data from the untargeted analysis to build separate linear classifier models that would distinguish the control and ALD group.

We used ROC analysis to reveal 3-hydroxytetradecanedioic acid, isocitric acid, and sebacic acid to be potent discriminators of the between control and ALD groups. We found that these biomarkers were linked with the breakdown of citrate cycle (TCA cycle), glyoxylate and dicarboxylate metabolism, and may give rise to subtle and early changes. Taking these biomarkers as screening indexes, the biomarker panel was validated in a cohort of normal control subjects and subjects with ALD with an AUC of >95%. To our knowledge, this is the first published report of a urine-based biomarker panel with very high accuracy for detecting preclinical ALD. Here we present the discovery and validation of urine metabolite changes that distinguish normal participants from ALD in the near future. The accuracy for detection is high, and urine is easier to obtain and costs less to acquire, making it more useful for screening in large-scale clinical trials and for future clinical use. This biomarker panel requires external validation using similar rigorous clinical classification before further development for clinical use.

In this study, a LC/MS urine metabolomics has been successfully established for biomarker studies in ALD. Here, we describe our metabolomics approach to detecting ALD disease in a group of 206. In conclusion, a total 6 urinary differential metabolites were identified and contributed to ALD progress, and more important, we discovered and validated 3 of them from urine with over 95% accuracy. The biomarker panel may be sensitive to early diagnosis of ALD disease. This is the first published report of a urine-based biomarker panel with very high accuracy for detecting ALD.

Abbreviations:

ALD, Alcoholic liver disease; OPLS, Orthogonal Projection to Latent Structures; PCA, Unsupervised' Principal Component Analysis; PLS-DA, supervised Partial Least Squares-Discriminant Analysis

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Conflict of interest:

The authors declare no competing financial interests.

References

1. George O, Sanders C, Freiling J, Grigoryan E, Vu S, Allen CD, Crawford E, Mandyam CD, Koob GF. Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking. Proc Natl Acad Sci U S A. 2012;109(44):18156-61.

2. Nasrallah NA, Clark JJ, Collins AL, Akers CA, Phillips PE, Bernstein IL. Risk preference following adolescent alcohol use is associated with corrupted encoding of costs but not rewards by mesolimbic dopamine. Proc Natl Acad Sci U S A. 2011;108(13):5466-71.

3. Kendrick SF, O'Boyle G, Mann J, Zeybel M, Palmer J, Jones DE, Day CP. Acetate, the key modulator of inflammatory responses in acute alcoholic hepatitis. Hepatology. 2010;51(6):1988-97.

4. Mookerjee RP, Malaki M, Davies NA, Hodges SJ, Dalton RN, Turner C, Sen S, Williams R, Leiper J, Vallance P, Jalan R. Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. Hepatology. 2007;45(1):62-71.

5. Kim WR, Gross JB Jr, Poterucha JJ, Locke GR 3rd, Dickson ER. Outcome of hospital care of liver disease associated with hepatitis C in the United States. Hepatology. 2001;33(1):201-6.

6. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, Nyati MK, Ahsan A, Kalyana-Sundaram S, Han B, Cao X, Byun J, Omenn GS, Ghosh D, Pennathur S, Alexander DC, Berger A, Shuster JR, Wei JT, Varambally S, Beecher C, Chinnaiyan AM (2009) Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature 457: 910-914.

7. Suhre K, Shin SY, Petersen AK, Mohney RP, Meredith D, Wägele B, Altmaier E; CARDIoGRAM, Deloukas P, Erdmann J, Grundberg E, Hammond CJ, de Angelis MH, Kastenmüller G, Köttgen A, Kronenberg F, Mangino M, Meisinger C, Meitinger T, Mewes HW, Milburn MV, Prehn C, Raffler J, Ried JS, Römisch-Margl W, Samani NJ, Small KS, Wichmann HE, Zhai G, Illig T, Spector TD, Adamski J, Soranzo N, Gieger C, Assimes TL, Deloukas P, Erdmann J, Holm H, Kathiresan S, König IR, McPherson R, Reilly MP, Roberts R, Samani NJ, Schunkert H, Stewart AF. (2011) Human metabolic individuality in biomedical and pharmaceutical research. Nature 477: 54-60.

8. Liang Q, Wang C, Li B, Zhang AH. Metabolic fingerprinting to understand therapeutic effects and mechanisms of silybin on acute liver damage in rat. Pharmacogn Mag. 2015;11(43):586-93.

9. Arakaki AK, Skolnick J, McDonald JF. Marker metabolites can be therapeutic targets as well. Nature. 2008;456(7221):443.

10. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.

11. Patti GJ, Yanes O, Shriver LP, Courade JP, Tautenhahn R, Manchester M, Siuzdak G. Metabolomics implicates altered sphingolipids in chronic pain of neuropathic origin. Nat Chem Biol. 2012;8(3):232-4.

12. Tomita M, Kami K. Cancer. Systems biology, metabolomics, and cancer metabolism. Science. 2012;336(6084):990-1.

13. Ioannidis JP, Khoury MJ. Improving validation practices in "omics" research. Science. 2011;334(6060):1230-2.

14. Liang Q, Wang C, Li B. Metabolomic Analysis Using Liquid Chromatography/Mass Spectrometry for Gastric Cancer. Appl Biochem Biotechnol. 2015;176(8):2170-84.

15. Mapstone M, Cheema AK, Fiandaca MS, Zhong X, Mhyre TR, Macarthur LH, Hall WJ, Fisher SG, Peterson DR8, Haley JM, Nazar MD, Rich SA, Berlau DJ, Peltz CB, Tan MT, Kawas CH, Federoff HJ. Plasma phospholipids identify antecedent memory impairment in older adults. Nat Med. 2014;20(4):415-8.

16. Manna SK, Patterson AD, Yang Q, Krausz KW, Idle JR, Fornace AJ, Gonzalez FJ. UPLC-MS-based urine metabolomics reveals indole-3-lactic acid and phenyllactic acid as conserved biomarkers for alcohol-induced liver disease in the Ppara-null mouse model. J Proteome Res. 2011;10(9):4120-33.

17. Zhang AH, Sun H, Han Y, Yan GL, Yuan Y, Song GC, Yuan XX, Xie N, Wang XJ. Ultraperformance liquid chromatography-mass spectrometry based comprehensive metabolomics combined with pattern recognition and network analysis methods for characterization of metabolites and metabolic pathways from biological data sets. Anal Chem. 2013;85(15):7606-12.

18. Kotz KT, Xiao W, Miller-Graziano C, Qian WJ, Russom A, Warner EA, Moldawer LL, De A, Bankey PE, Petritis BO, Camp DG 2nd, Rosenbach AE, Goverman J, Fagan SP, Brownstein BH, Irimia D, Xu W, Wilhelmy J, Mindrinos MN, Smith RD, Davis RW, Tompkins RG, Toner M; Inflammation and the Host Response to Injury Collaborative Research Program. Clinical microfluidics for neutrophil genomics and proteomics. Nat Med. 2010;16(9):1042-7.

19. Zhang A, Sun H, Han Y, Yan G, Wang X. Urinary metabolic biomarker and pathway study of hepatitis B virus infected patients based on UPLC-MS system. PLoS One. 2013;8(5):e64381

20. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.

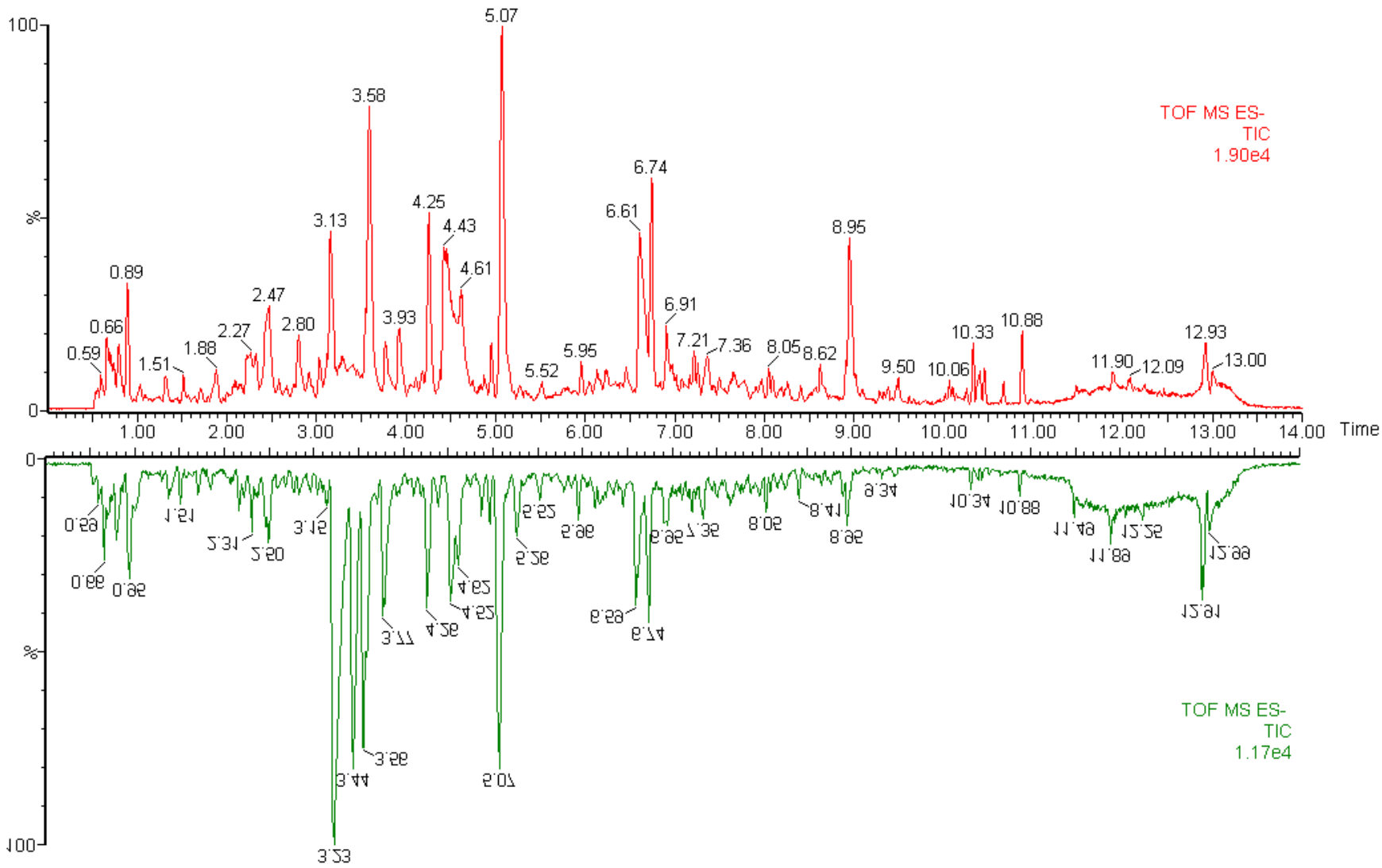

Fig. 1. Typical total ion current chromatograms of control subjects (up) and ALD patients (down).

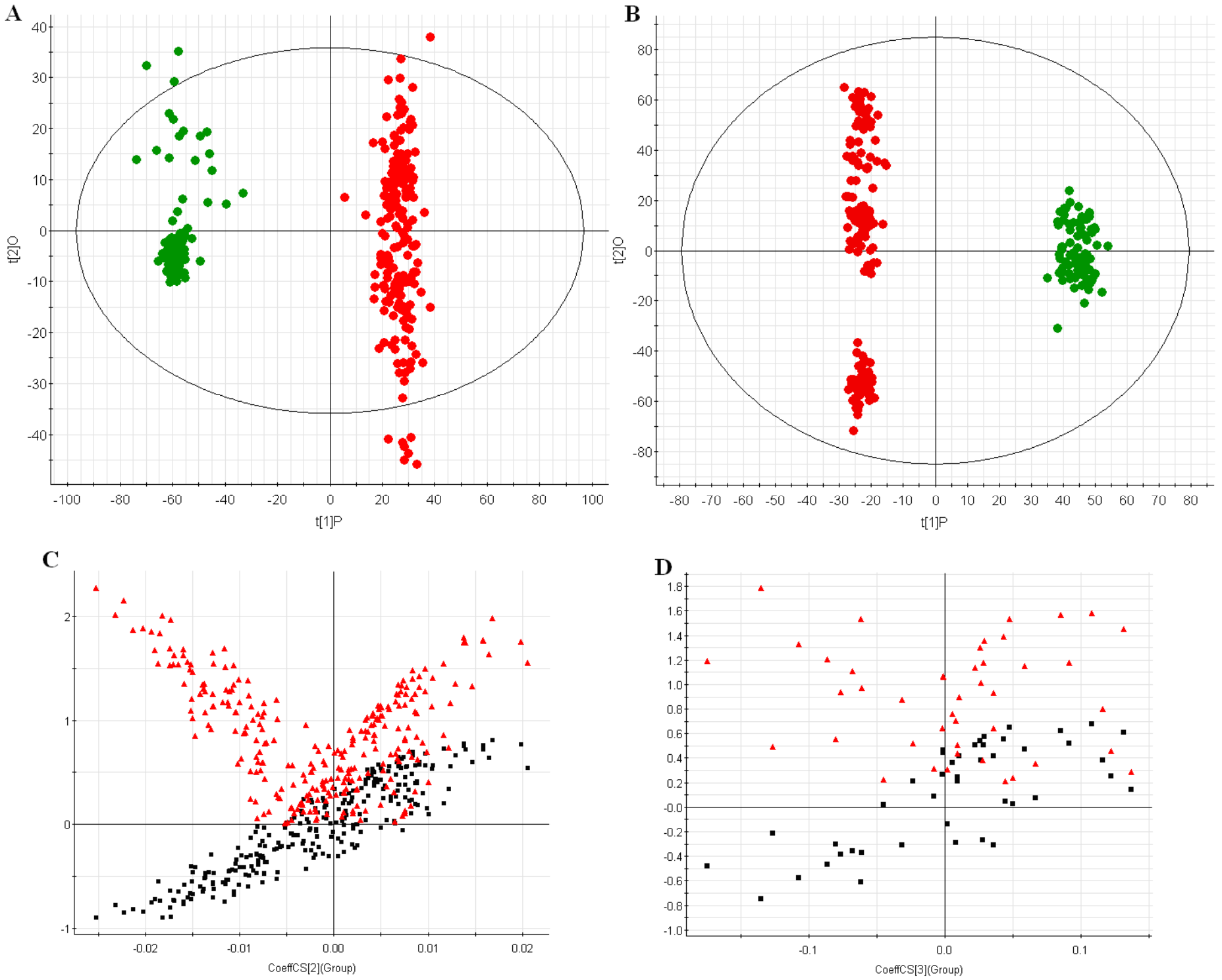

Fig. 2. Metabolomic profiling of alcoholic liver fibrosis. PCA score plots of urine samples collected from normal (green), ALD (red) groups in positive ion mode (A) and negative ion mode (B). Panel C and D show the combination of VIP-score plots constructed from the supervised OPLS-DA analysis of urine in positive mode and negative ion mode, respectively.

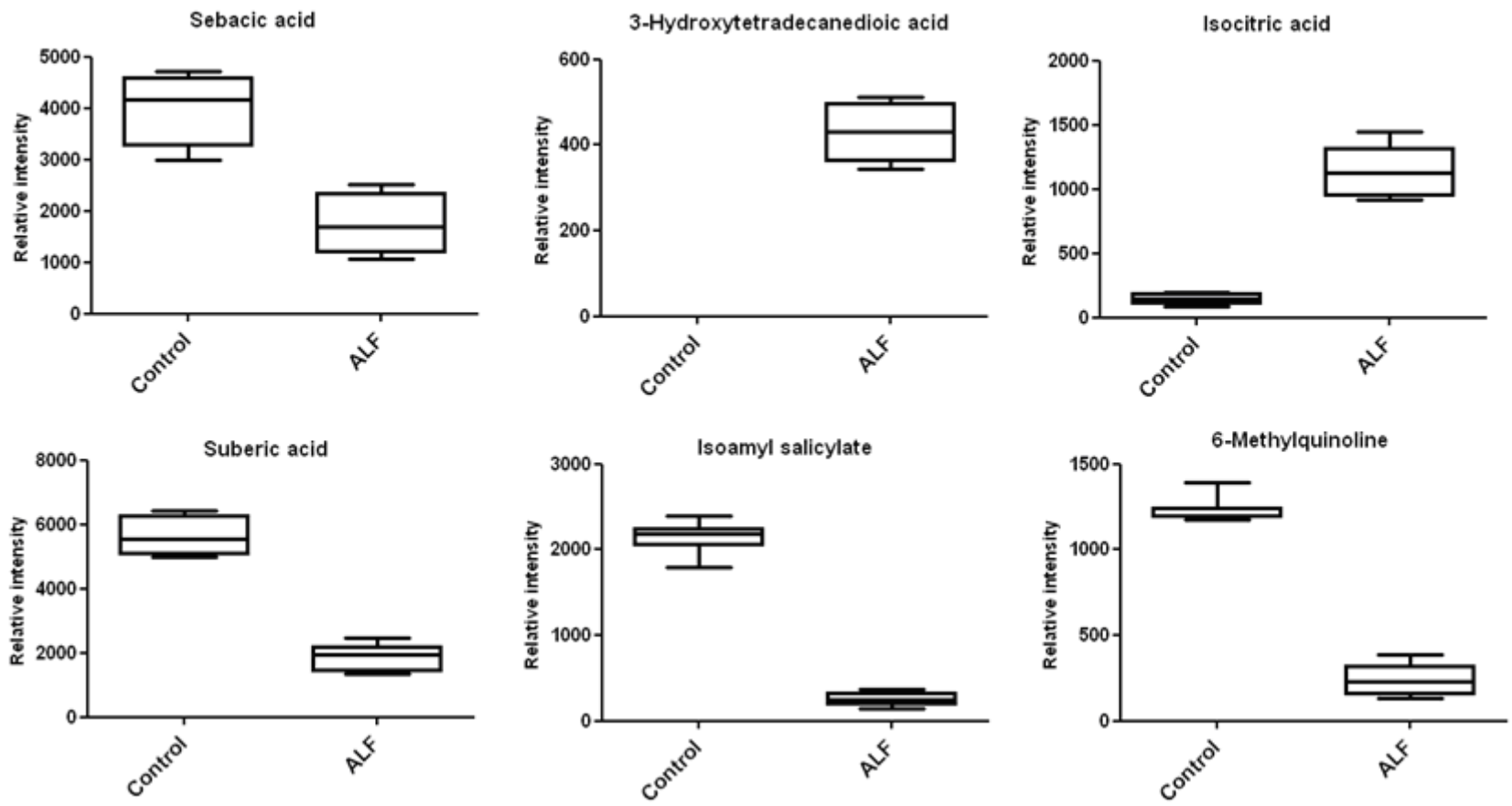

Fig. 3 Box plots show UPLC-MS relative signal intensities for metabolites in control and ALD groups. The targeted analysis of the six metabolites in the discovery phase and the application of the metabolite panel developed from the targeted discovery phase in the independent validation phase.

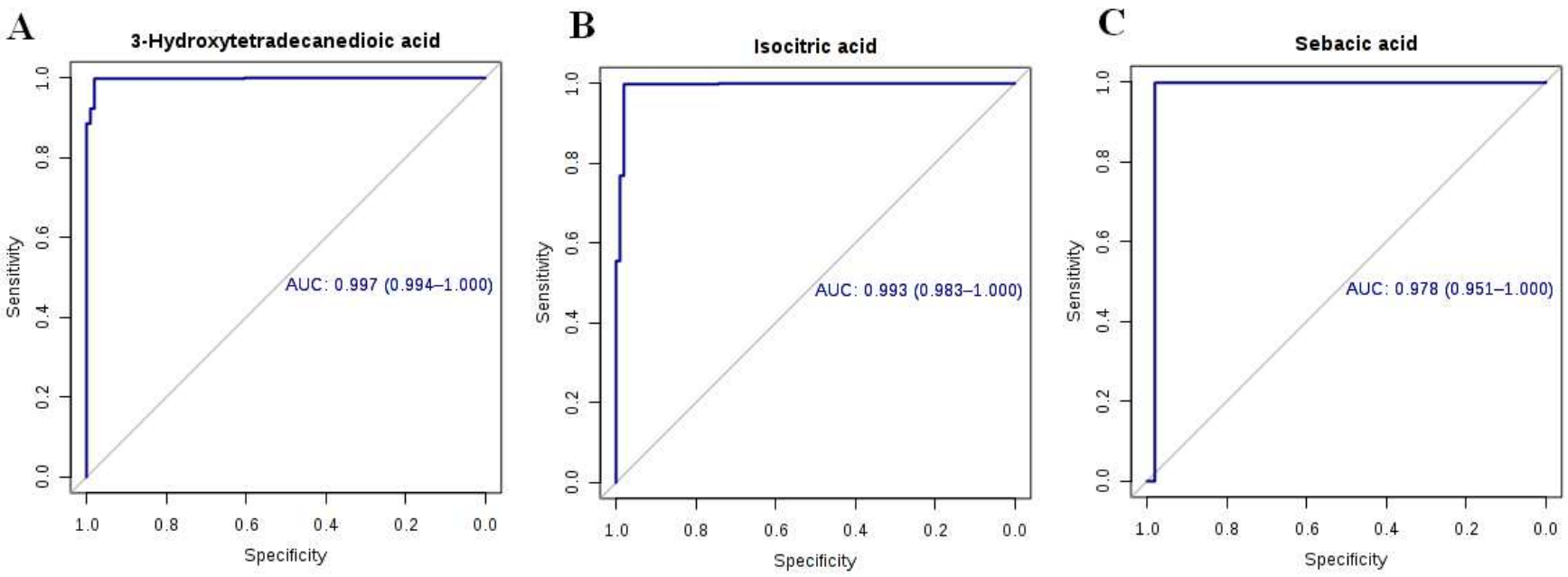

Fig. 4 ROC results for the metabolomics analyses.

ROC plots represent sensitivity versus 1 – specificity, and the blue line represents the AUC.

Table 1. Putative metabolite markers resulting from ALD in positive and negative mode.

No	Compound ID	Adducts	Formula	Mass Error (ppm)	m/z	Retention time (min)	Compound	Trend	VIP
1	HMDB00792	M-H	C10H18O4	-0.42482	201.1126	4.947166667	Sebacic acid	↓	1.7847
2	HMDB00394	M-H	C14H26O5	3.431132	273.1711	7.0795	3-Hydroxytetradecanedioic acid	↑	1.5822
3	HMDB00193	M-H	C6H8O7	-4.35433	191.0183	0.780166667	Isocitric acid	↑	1.5375
4	HMDB00893	M-H	C8H14O4	2.088976	173.0817	5.322166667	Suberic acid	↑	1.5035
5	HMDB40225	M+H	C12H16O3	-1.57926	209.1174	7.189166667	Isoamyl salicylate	↓	2.2786
6	HMDB33115	M+H	C10H9N	-3.11727	144.0809	2.340266667	6-Methylquinoline	↑	2.1561

Note: The markers were chosen on the basis of significant predictive value as determined by VIP-score plots constructed from the supervised OPLS-DA. Arrows indicate upregulation or downregulation in the comparison group as compared to the control participants.

Supplementary Table 1. Clinical characteristics of the subjects at baseline

Samples	ALD	Control
Sample No.	206	101
Age	31±3	30±2
Sex (F/M)	198/318	46/55
BMI(kg/m2)	26.11±2.54	23.24±2.19
ALT (U/L)	146.33±68.35	89.17±56.40
AST (U/L)	166.87±84.43	96.10±67.45
Total bilirubin (mg/dL)	4.29±2.47	1.91±1.04
Direct bilirubin(mg/dL)	2.81±1.67	1.34 ± 0.88
Indirect bilirubin(mg/dL)	1.92±0.46	0.89 ± 0.29