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World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1375 experts in gastroenterology and hepatology from 68 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents[®]/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch[®]), Journal Citation Reports[®], Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. The 2015 edition of Journal Citation Reports[®] released by Thomson Reuters (ISI) cites the 2015 impact factor for *WJG* as 2.787 (5-year impact factor: 2.848), ranking *WJG* as 38 among 78 journals in gastroenterology and hepatology (quartile in category Q2).

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I-IX Editorial Board

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLISHER

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7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
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PUBLICATION DATE
April 21, 2017

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Clinical Trials Study

Urinary metabolomics analysis identifies key biomarkers of different stages of nonalcoholic fatty liver disease

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Supported by the Major Project of Shanghai Municipal S&T Commission, No. 15DZ1900104; and National Natural Science Foundation of China, No. 81273729.

Institutional review board statement: The study protocol was approved by the Ethics Review Committee of the Faculty of Medicine, Shuguang Hospital, China, and was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice Guidelines.

Clinical trial registration statement: This study has been registered on ClinicalTrials.gov. Identifier No. NCT02077283.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest regarding the publication of this paper.

Data sharing statement: Readers can obtain the information of the study and the original data by contacting the author Shu Dong at lisadongshu@163.com.

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Manuscript source: Unsolicited manuscript

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Received: December 5, 2016

Peer-review started: December 6, 2016

First decision: December 29, 2016

Revised: January 13, 2017

Accepted: February 17, 2017

Article in press: February 17, 2017

Published online: April 21, 2017

Abstract

AIM

To identify a panel of biomarkers that can distinguish between non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), and explore molecular mechanism involved in the process of developing NASH from NAFLD.

METHODS

Biomarkers may differ during stages of NAFLD. Urine and blood were obtained from non-diabetic subjects with NAFLD and steatosis, with normal liver function ($n = 33$), from patients with NASH, with abnormal liver function ($n = 45$), and from healthy age and sex-

matched controls ($n = 30$). Samples were subjected to metabolomic analysis to identify potential non-invasive biomarkers. Differences in urinary metabolic profiles were analyzed using liquid chromatography tandem mass spectrometry with principal component analysis and partial least squares-discriminate analysis.

RESULTS

Compared with NAFLD patients, patients with NASH had abnormal liver function and high serum lipid concentrations. Urinary metabolomics found differences in 31 metabolites between these two groups, including differences in nucleic acids and amino acids. Pathway analysis based on overlapping metabolites showed that pathways of energy and amino acid metabolism, as well as the pentose phosphate pathway, were closely associated with pathological processes in NAFLD and NASH.

CONCLUSION

These findings suggested that a panel of biomarkers could distinguish between NAFLD and NASH, and could help to determine the molecular mechanism involved in the process of developing NASH from NAFLD. Urinary biomarkers may be diagnostic in these patients and could be used to assess responses to therapeutic interventions.

Key words: Urinary metabolomics; Nonalcoholic fatty liver disease; Steatohepatitis

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Core tip: To identify biomarkers that can distinguish between nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), urine and blood were obtained from patients with NAFLD and NASH, and healthy controls. Urinary metabolomics found differences in 31 metabolites between NAFLD and NASH, including nucleic acids and amino acids. Pathway analysis showed that pathways of energy metabolism, amino acid metabolism, and the pentose phosphate pathway, were closely associated with the pathological processes in NAFLD and NASH. These biomarkers could distinguish between NAFLD and NASH, and could help to determine the mechanism involved in the development of NASH from NAFLD.

Dong S, Zhan ZY, Cao HY, Wu C, Bian YQ, Li JY, Cheng GH, Liu P, Sun MY. Urinary metabolomics analysis identifies key biomarkers of different stages of nonalcoholic fatty liver disease. *World J Gastroenterol* 2017; 23(15): 2771-2784 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i15/2771.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i15.2771>

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) comprises

a spectrum of pathological conditions, including simple steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis. NAFLD has been estimated to affect approximately 15%-30% of the general population and its prevalence is increasing worldwide^[1,2]. The prevalence of NAFLD is strongly linked to obesity, insulin resistance, and a cluster of metabolic disorders, including hypertriglyceridemia and hyperuricemia^[3], which impair health seriously^[4].

No standard treatment exists currently to manage NAFLD, or even NASH, in western medicine^[5]. Weight loss regimens, including restricted calorie diets, bariatric surgery, and drug-induced fat malabsorption, only improve the condition to some degree^[6-8]. Identification of metabolic differences among the stages of NAFLD might result in the development of more effective and specific treatments for NAFLD and NASH.

Urine metabolomics^[9] is a good method to assess metabolic differences among different stages of NAFLD. Although urinary metabolomics data have been obtained in patients with NAFLD, NASH, and liver cirrhosis^[10], to date, few studies have used this method to compare patterns in patients at different stages of NAFLD. This study aimed to investigate correlations between disease stages and urine metabolomics in patients with NAFLD, specifically to determine whether urine metabolomics could be used to distinguish NAFLD from NASH. In addition, this study sought to determine the molecular mechanisms involved in the development of NASH from NAFLD.

MATERIALS AND METHODS

Population, information, and sample collection

The randomized clinical trial evaluated patients seen at the NAFLD outpatient clinic of Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine from January 2013 to May 2014. Healthy volunteer controls were enrolled from among employees of the medical center. Figure 1 provides an overview of the study. A total of 108 subjects were recruited, 33 in the NAFLD group, which included patients with steatosis and normal liver function; 45 in the NASH group, which included patients with steatohepatitis and abnormal liver function; and 30 healthy controls. All subjects provided written informed consent.

General information recorded at each participant's first visit to a doctor included age, gender, and medical history. The results of laboratory tests and ultrasound were also recorded. Urine samples for metabolic profiling were collected from participants at their second visit.

Diagnostic criteria for NAFLD and NASH

The diagnostic criteria for NAFLD included: (1) a history of no or limited daily alcohol intake (< 20 g for women and < 30 g for men); (2) the presence of hepatic steatosis by imaging or histology; and (3) the

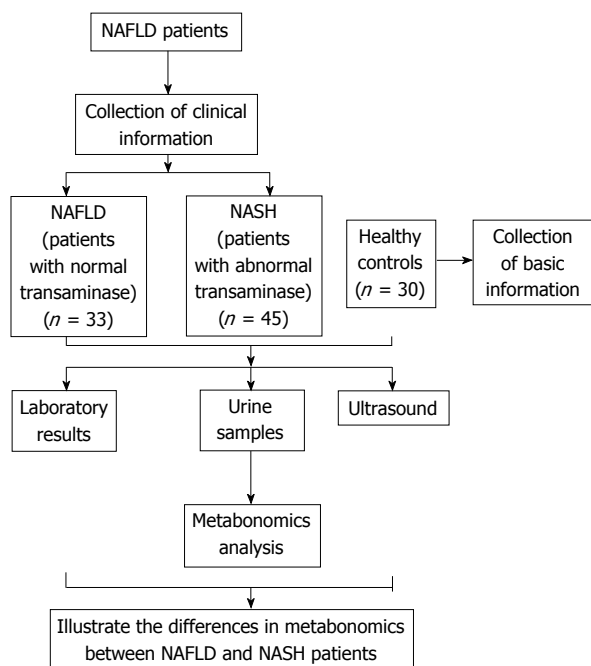


Figure 1 Flow diagram of the study protocol. NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

exclusion of all other liver diseases^[11]. The diagnostic criteria for NASH included: (1) a diagnosis of NAFLD, as above; and (2) a significant increase in alanine aminotransferase (ALT) activity or other liver function parameters.

Inclusion criteria

Males and females aged 18-60 years, without medication, were eligible following a screening test to confirm the presence of NAFLD. Based on their symptoms and the results of liver function tests, NAFLD patients were divided into NAFLD and NASH groups, consisting of patients with normal and abnormal liver function, respectively.

Exclusion criteria

Patients were excluded if they: (1) had a history of diabetes mellitus or any metabolic disease; (2) consumed > 20 g alcohol per day; (3) had acute diseases or other untreated illness requiring treatment; (4) had impaired hepatic or renal functions; (5) were female of childbearing age who were pregnant, lactating, or unwilling to use an effective form of birth control; (6) had medication or other treatment before; or (7) had a history or presence of any condition that, in the investigator's opinion, would endanger the individual's safety or affect the study results.

Urine sample collection and handling

Urine samples were collected from each participant during mid-morning and were centrifuged at 4 °C for 15 min at 1509.3 × *g*. The supernatants were frozen and stored at -80 °C until analysis. If required, urine

samples were transported using Drikold.

Liquid chromatography-mass spectrometry analysis

Pretreatment: 100 µL of urine and 300 µL of acetonitrile were vortexed for 3 min and then centrifuged at 12000 r/min, 4 °C for 10 min. Supernatants were kept as prepared samples for further analysis.

Liquid chromatography (LC) separation was performed on an Agilent 1200 series LC system (Agilent, CA, United States). Aliquots of 2 µL of the prepared samples were injected into a Waters Shield C18 column (3.5 µm, 2.1 mm × 150 mm) maintained at 20 °C, and eluted with a mobile phase of 0.01% formic acid in water-acetonitrile (90:10) at a flow rate of 0.3 mL/min. MS detection was performed on an API 4000 triple quadrupole mass spectrometer (Sciex Applied Biosystems), using positive electronic spray ionization in multiple reaction monitoring mode, and at a source temperature of 700 °C and a voltage of 5500 V. The dwell time for the multiple reaction monitoring mode was 0.08 s. Nitrogen was used as the curtain, nebulizer, and collision gas, at pressures of 50, 60, and 70 psi, respectively. Certain ion transitions for amino acids and their internal standards were monitored, and peak area ratios of amino acids to internal standards were calculated after correcting for transition overlaps of natural leucine and isoleucine^[12].

Metabolite identification

Compounds were identified by comparison with library entries of purified standards and recurrent unknown entities. Known chemical entities were identified based on comparisons with metabolomic library entries of more than 2362 commercially available purified standards and an online database (<http://metlin.scripps.edu/>). In addition, currently unknown entities were identified by their recurrent nature^[13].

Statistical analysis

Data were analyzed by parametric and nonparametric statistical tests using SPSS (version 16) and Simca-P (version 11.0). Continuous data were compared by one-way ANOVA. Differences in metabolic profiles on LC/MS were determined by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA).

To validate the importance of the metabolites, and to further gauge their ability to distinguish among patients with NASH and NAFLD and healthy controls, their potential predictive utility for the process of NAFLD was assessed by receiver operating characteristic (ROC) curve analysis. ROC analysis was performed using MS peak areas corresponding to the metabolite concentrations in each of the three subject groups. Areas under the ROC curve were calculated using the ROCR package (classifier visualization in R).

Quality control

The measurements from each patient's laboratory

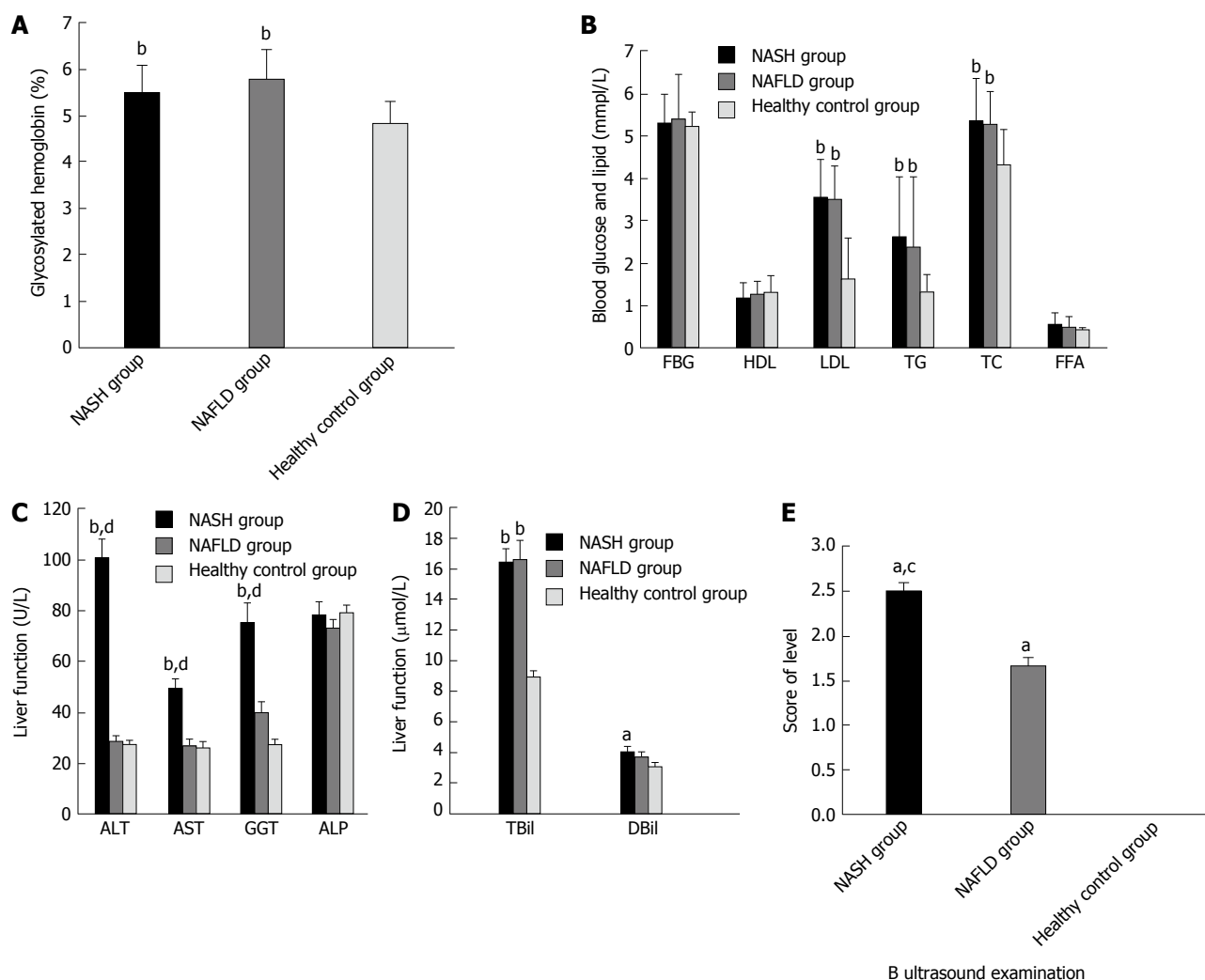


Figure 2 Characteristics of the study participants. Mean concentrations of (A) glycosylated hemoglobin (HbA1c); (B) fasting blood glucose (FBG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), total cholesterol (TC); (C) alanine aminotransferase (ALT), and aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alpha-fetoprotein (AFP); and (D) total bilirubin (TBil) and direct bilirubin (DBil) in the non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and healthy control groups. E: Results of ultrasound examination in the three groups. Significant differences among the three groups were assessed by one-way ANOVA in A-D and by *t*-tests in E. ^a*P* < 0.05, ^b*P* < 0.01 vs the control group; ^c*P* < 0.05, ^d*P* < 0.01 vs the NAFLD group.

test results were entered into an Excel spreadsheet, followed by re-checking of all data to ensure accuracy.

RESULTS

Characteristics of study participants

There were no significant differences among the three groups in terms of patient number, age, and height. Weight and body mass index (BMI) were significantly higher in the NAFLD and NASH groups compared with those in the control group (Table 1).

Compared with the healthy group, patients with NAFLD and NASH had much higher concentrations of glycosylated hemoglobin (HbA1c), low-density lipoprotein-cholesterol (LDL), triglycerides (TG), total cholesterol (TC), ALT, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and total bilirubin (TBil). Compared with the NAFLD group, patients with NASH had much higher ALT, AST, and

GGT concentrations, and significantly different results on ultrasound examinations (Table 2 and Figure 2).

Urine metabonomics

PCA was performed using samples from the three groups. S-plots showed obvious metabolic differences among these three groups (Figure 3). This was followed by pair-wise comparisons.

NAFLD group vs control group: Urinary metabonomics were used to assess differences between the NAFLD and control groups. PCA showed a spectral separation between these two groups, indicating significant metabolic differences. This was further supported by PLS-DA and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) (Figure 4). After filtering out interference signals, 53 different metabolites were detected; mainly nucleic acids and amino acids (Table 3). The concentrations of

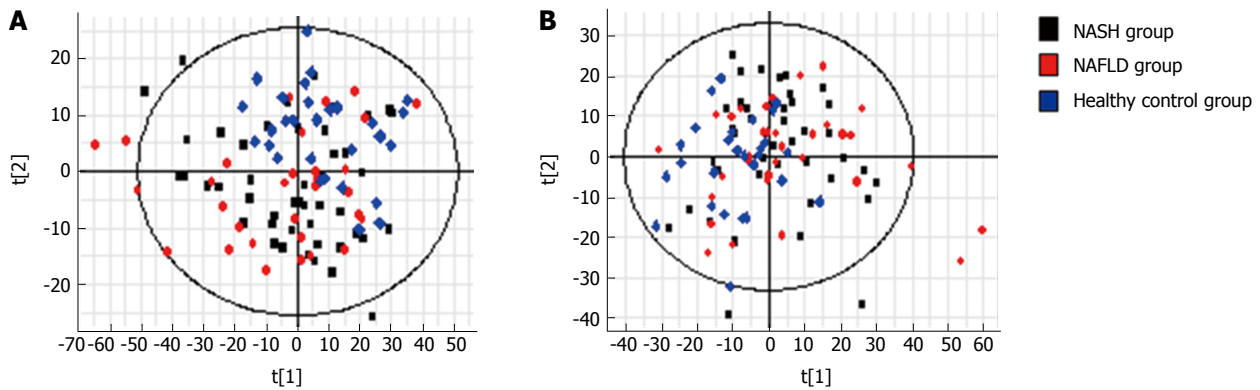


Figure 3 S-plots of PCA analysis (A) with electrospray ionization (ESI+) and (B) without electrospray ionization (ESI-) in the non-alcoholic steatohepatitis, non-alcoholic fatty liver disease, no comma symbol and control groups. PCA: Principal component analysis; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

Table 1 Demographic and clinical characteristics of the study participants (mean \pm SD)

Group	NASH	NAFLD	Healthy group
Number	45	33	30
Age (yr)	39 \pm 10	44 \pm 14	39 \pm 4
Gender	Male (38) Female (7)	Male (19) Female (14)	Male (21) Female (9)
Height (cm)	169 \pm 8	170 \pm 6	168 \pm 6
Weight (kg)	76 \pm 13 ^b	78 \pm 11 ^b	68 \pm 9
BMI (kg/m ²)	26.40 \pm 3.49 ^b	26.81 \pm 3.43 ^b	23.73 \pm 1.95

^b*P* < 0.01 vs the healthy group. NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

the nucleic acid metabolites hypoxanthine, xanthine, and carnitine were lower in the urine of patients with NAFLD than in the control subjects. In addition, the concentrations of the amino acid metabolites, citrulline, arginine, valine, and indole acetic acid, as well as glucose and gluconic acid, were higher in patients with NAFLD than in the controls. ROC analysis, performed to identify the key metabolites that could distinguish NAFLD patients from healthy individuals, found that 7-methylxanthine, 2-methylguanosine, gluconic acid, and indoxylsulfuric acid were markers for NAFLD (Figure 5).

NASH group vs control group: Urinary metabolomics were also used to assess differences between the NASH and control groups. PCA analysis showed obvious spectral separation between the two groups, indicative of significant metabolic differences between NASH patients and healthy controls. This was further supported by PLS-DA and OPLS-DA (Figure 6). After filtering out the interference signals, 88 different metabolites (Table 4) were detected, consisting mainly of amino acids and their metabolic intermediates. Compared with the healthy controls, patients with NASH had much higher urinary levels of lysine, valine, citrulline, arginine, threonine, tyrosine, leucine, hippuric acid, and 3-indoleacetic acid, and lower levels

Table 2 Laboratory test results in the three groups of study of participants (mean \pm SD)

Group	NASH	NAFLD	Healthy group
HbA1C (%)	5.51 \pm 0.58 ^b	5.8 \pm 0.62 ^b	4.83 \pm 0.47
FBG (mmol/L)	5.31 \pm 0.69	5.4 \pm 1.05	5.25 \pm 0.33
HDL (mmol/L)	1.2 \pm 0.36	1.28 \pm 0.3	1.33 \pm 0.37
LDL (mmol/L)	3.57 \pm 0.88 ^b	3.53 \pm 0.77 ^b	1.64 \pm 0.95
TG (mmol/L)	2.62 \pm 1.4 ^b	2.39 \pm 1.64 ^b	1.32 \pm 0.41
TC (mmol/L)	5.37 \pm 0.98 ^b	5.3 \pm 0.77 ^b	4.32 \pm 0.83
FFA (mmol/L)	0.58 \pm 0.25	0.5 \pm 0.24	0.44 \pm 0.05
ALT (U/L)	100.66 \pm 48.4 ^{bd}	28.9 \pm 10.76	27.36 \pm 9.76
AST (U/L)	49.68 \pm 23.1 ^{bd}	27.17 \pm 12.74	26.41 \pm 13.05
GGT (U/L)	75.26 \pm 53.1 ^{bd}	40.25 \pm 23.66	27.39 \pm 12.04
ALP (U/L)	78.5 \pm 33.31	73.43 \pm 17.05	79.1 \pm 16.02
Tbil (μ mol/L)	16.42 \pm 6.24 ^b	16.63 \pm 7.04 ^b	8.99 \pm 1.92
DBil (μ mol/L)	4.07 \pm 2.18	3.7 \pm 1.72	3.05 \pm 1.34
B ultrasound examination	2.51 \pm 0.55 ^{ac}	1.67 \pm 0.48 ^a	0 \pm 0

^a*P* < 0.05, ^b*P* < 0.01 vs the healthy group; ^c*P* < 0.05, ^d*P* < 0.01 vs the NAFLD group. NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease; HbA1c: Glycosylated hemoglobin; FBG: Fasting blood glucose; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglycerides; TC: Total cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase; Tbil: Total bilirubin; Dbil: Direct bilirubin.

of derivatives of indole acetic acid, such as 5-hydroxy indole acetic acid and indole-3-formic acid. In addition, cortisol levels decreased significantly. ROC analysis showed that 2-methylguanosine, gluconic acid, indoxylsulfuric acid, cAMP, indolelactic acid, and acetyl-DL-leucine could distinguish patients with NASH from healthy individuals (Figure 7).

NAFLD group vs NASH group: Metabolic PCA analysis of urine samples from patients with NAFLD and NASH showed spectral separation between the two groups of samples, indicating significant metabolic differences. OPLS-DA was performed to better assess these differences (Figure 8). After filtering out interference signals, 31 different metabolites (Table 5) were detected, mainly nucleic acids and amino acids. Compared with the NAFLD group, patients with NASH

Table 3 Urinary metabolites differentially expressed in non-alcoholic fatty liver disease patients and healthy controls

No.	Metabolites	VIP-value (OPLS-DA)	P value (t-test)	Fold change
ESI+				
1	L-Carnitine	1.488	0.002	1.531
2	Creatinine	1.195	0.015	0.257
3	L-Valine/betaine	1.195	0.015	-0.544
4	Acetylcarnitine	1.658	0.001	1.669
5	N α -Acetyl-L-arginine	1.297	0.008	-0.350
6	Hypoxanthine	1.883	0.000	0.968
7	1-Methylguanine	1.376	0.005	0.483
8	Adipic acid	1.534	0.001	-0.627
9	Xanthosine	1.467	0.002	0.389
10	Guanosine	1.448	0.003	0.273
11	7-Methylxanthine	1.489	0.002	1.922
12	2-Methylguanosine	1.654	0.001	0.475
13	Butyryl-L-carnitine	1.499	0.002	0.560
14	Gluconic acid	1.391	0.004	-0.733
15	Xanthurenic acid	1.351	0.006	0.485
16	Kynurenic acid	1.590	0.001	0.560
17	Indole-3-carboxylic acid	1.189	0.015	0.496
18	6 β -hydroxytestosterone	2.203	0.000	1.251
19	Androstenedione	1.500	0.002	0.779
20	PGA2 methyl ester	1.709	0.000	0.676
21	Cortisol	1.340	0.006	0.641
22	Deoxycorticosterone	1.770	0.000	0.841
23	Corticosterone	1.568	0.001	0.766
24	Cortisone	1.383	0.004	0.661
25	Testosterone glucuronide	1.838	0.000	0.844
26	EPA	1.208	0.014	0.714
27	Decanoyl-L-carnitine	1.592	0.001	1.101
28	Androsterone	2.276	0.000	0.918
29	Eicosapentaenoic Acid ethyl ester	1.282	0.009	-0.417
30	Ursodeoxycholic acid	1.599	0.001	0.474
ESI-				
31	Shikimate-3-phosphate	1.195	0.020	-0.344
32	2-keto-D-gluconic acid	1.902	0.000	-0.426
33	α -D-glucose	1.647	0.001	-0.484
34	Pyroglutamic acid	1.545	0.002	0.334
35	(S)-2-hydroxyglutarate	1.299	0.011	-0.579
36	2-Deoxy-D-ribose	1.264	0.014	-0.455
37	1-Methyluric acid	1.181	0.022	0.870
38	Salicyluric acid	1.455	0.004	-0.723
39	Salicylic acid	1.170	0.023	-0.494
40	Indoxylsulfuric acid	1.829	0.000	0.646
41	Ferulic acid 4-O-glucuronide	2.046	0.000	-2.303
42	Caffeic acid 3-sulfate	1.320	0.010	-2.214
43	2,3-Dihydroxybenzoic acid	1.857	0.000	-1.004
44	3,3-Dimethylglutaric acid	1.635	0.001	-1.225
45	Ferulic acid 4-sulfate	1.695	0.001	-1.779
46	Deoxyinosine	1.315	0.010	-1.049
47	Indolelactic acid	1.251	0.015	-0.868
48	3-Methylsuberic acid	1.577	0.002	-1.825
49	L-Homocitrulline	1.619	0.001	-1.883
50	Glycocholic acid	1.187	0.021	0.408
51	Glycoursodeoxycholic acid	1.392	0.006	0.932
52	L-homotyrosine	1.475	0.004	-0.625
53	Ethisterone	1.702	0.001	-0.659

had much higher concentrations of methyl xanthine, tryptophan, 3-indole acetic acid, and gluconic acid, and a lower level of proline. ROC analysis showed that 3-indoleacetic acid, L-carnitine, pyroglutamic acid, and indolelactic acid could distinguish NASH from NAFLD samples (Figure 9).

Key differential metabolites among the NAFLD, NASH, and control groups: The differentially expressed metabolites in the three pairwise comparisons were combined to determine the metabolites that overlapped in the three groups. Seven metabolites were screened through Venn analysis: L-carnitine, acetyl

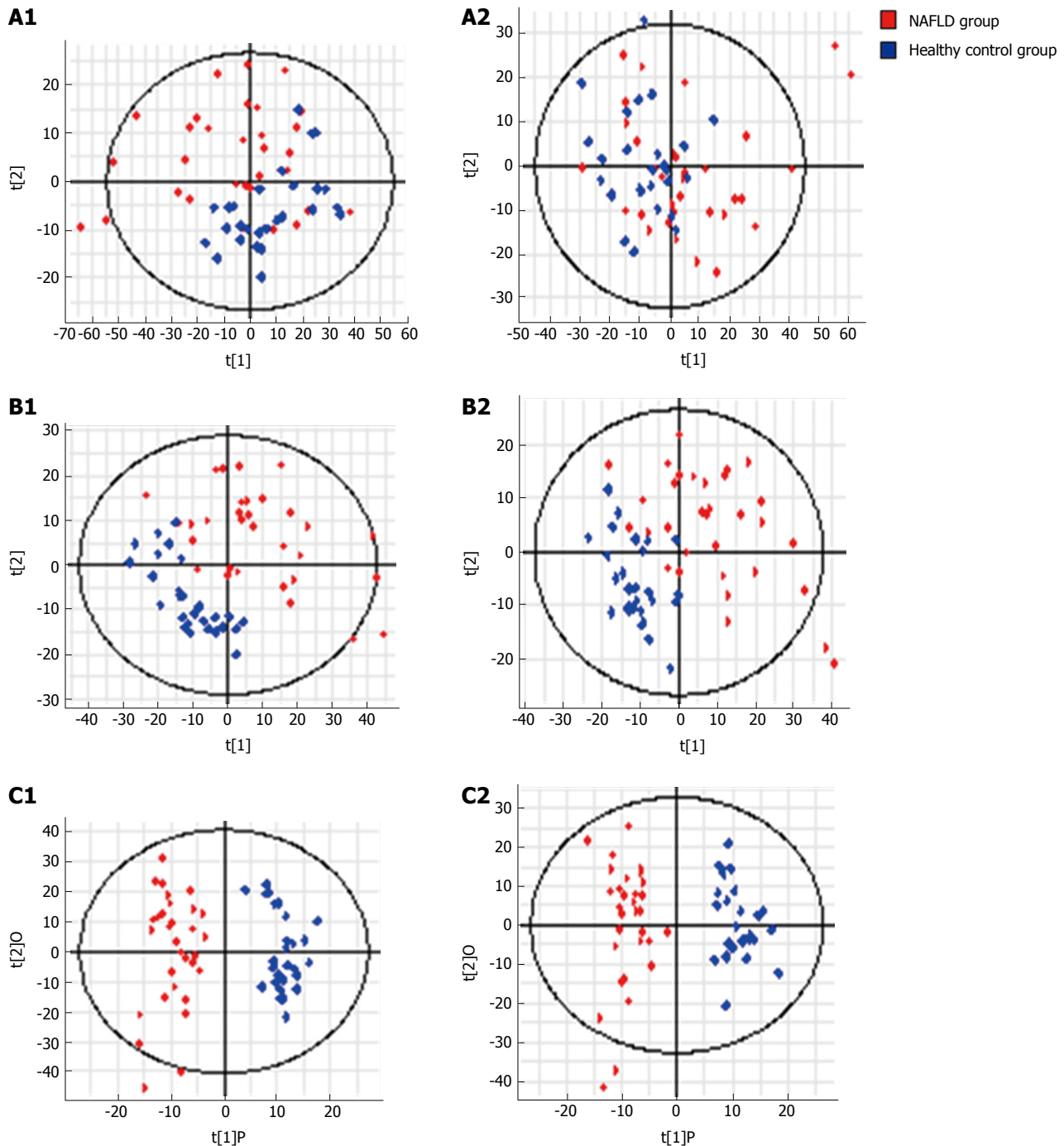


Figure 4 S-plots following (A) PCA, (B) PLS, and (C) OPLS analyses with (A1, B1 and C1) electrospray ionization (ESI+) and without (A2, B2 and C2) electrospray ionization (ESI-) in the non-alcoholic fatty liver disease and control groups. PCA: Principal component analysis; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

carnitine, gluconic acid, deoxycorticosterone, 2-keto-D-gluconic acid, pyroglutamic acid, and indolelactic acid (Figure 10). Kyoto Encyclopedia of Genes and genomes pathway analysis showed that these seven metabolites were enriched in seven pathways: metabolic pathways, the pentose phosphate pathway, antibiotic biosynthesis pathways antibiotics, steroid hormone biosynthesis, bile secretion, carbon metabolism, and glutathione metabolism. Three of these pathways, the pentose phosphate, carbon metabolism, and glutathione

metabolism pathways, might be associated closely with the pathological processes of NAFLD and NASH.

DISCUSSION

Obesity, insulin resistance, and associated metabolic perturbations are observed frequently in patients with NAFLD^[14,15]. NASH is a type of NAFLD with serious abnormalities in liver function^[16]. NAFLD has a significant impact on health, affecting many body systems^[17].

Table 4 Urinary metabolites differentially expressed in non-alcoholic steatohepatitis patients and healthy controls

No.	Metabolites	VIP-value (OPLS-DA)	P value (t-test)	Fold change
ESI+				
1	L-Lysine	1.291	0.007	-0.747
2	Suberic acid	1.037	0.032	-0.370
3	L-Carnitine	1.015	0.036	0.804
4	Creatinine	1.052	0.030	0.206
5	L-Valine/betaine	1.668	0.000	-0.852
6	Citrulline	1.539	0.001	-0.451
7	L-Dopa	1.249	0.009	-0.322
8	Acetylcarnitine	1.213	0.012	0.971
9	N α -Acetyl-L-arginine	1.128	0.019	-0.658
10	L-Threonine	1.009	0.037	-0.300
11	L-Tyrosine	1.217	0.011	-0.304
12	Uridine	1.106	0.022	0.114
13	Hypoxanthine	2.406	0.000	1.093
14	2'-O-Methyladenosine	1.345	0.005	0.222
15	1-Methylguanine	1.921	0.000	0.744
16	6-Hydroxynicotinic acid	1.617	0.001	-0.957
17	Adipic acid	1.635	0.001	-0.808
18	Glycerophosphocholine	1.623	0.001	0.299
19	cAMP	2.233	0.000	0.417
20	L-Proline	1.046	0.031	0.397
21	Dimethyl fumarate	1.716	0.000	-0.627
22	5-Hydroxy-L-tryptophan	1.118	0.021	0.190
23	Xanthosine	1.467	0.002	0.318
24	D-Ribose	0.994	0.040	0.141
25	2-Methylguanosine	2.145	0.000	0.528
26	Butyryl-L-carnitine	1.941	0.000	0.569
27	α -Hydroxyhippuric acid	1.134	0.019	-0.540
28	Gluconic acid	1.098	0.023	-0.537
29	N-Acetylproline	1.486	0.002	0.491
30	Kynurenic acid	1.543	0.001	0.465
31	Indoxylsulfuric acid	1.444	0.002	0.494
32	Ferulic acid	0.982	0.043	-0.948
33	5-Hydroxyindoleacetic acid	1.420	0.003	0.312
34	Acetyl-DL-leucine	1.419	0.003	-0.522
35	Indole-3-carboxylic acid	0.969	0.046	0.359
36	3-Indoleacetic acid	1.055	0.029	-0.458
37	6 β -Hydroxytestosterone	2.074	0.000	0.929
38	Estrone glucuronide	1.111	0.021	-1.448
39	PGA2 methyl ester	1.305	0.006	0.389
40	Cortisol	1.864	0.000	0.658
41	Tetrahydrocortisone	1.173	0.015	0.412
42	Corticosterone	1.165	0.016	0.406
43	Deoxycorticosterone	1.502	0.002	0.551
44	Cortisone	1.444	0.002	0.633
45	Ethisterone	1.541	0.001	0.689
46	EPA	0.953	0.050	0.456
47	Decanoyl-L-carnitine	1.295	0.007	0.828
48	Androsterone	2.370	0.000	0.741
49	Lauroylcarnitine	1.455	0.002	0.700
50	Palmitic amide	1.277	0.008	0.674
51	Stearamide	1.655	0.000	1.008
52	Ursodeoxycholic acid	1.231	0.010	0.398
ESI-				
53	N-Acetylneuraminic acid	0.993	0.034	-0.257
54	5-aminosalicylic acid	2.042	0.000	-0.877
55	Guanine	1.082	0.021	-0.369
56	p-Coumaric acid	1.315	0.004	-0.852
57	2-Keto-glutaramic acid	1.058	0.024	0.201
58	L-2-Aminoadipic acid	0.968	0.039	0.265
59	N-Acetyl-L-glutamic acid	1.046	0.026	0.248
60	Pyroglutamic acid	2.089	0.000	0.483
61	2-Deoxy-D-ribose	1.040	0.026	-0.672
62	N-Acetylaspartylglutamic acid	1.091	0.020	0.250
63	(S)-2-Hydroxyglutarate	1.512	0.001	-0.754
64	Vanillylmandelic acid	1.065	0.023	0.269

65	(S)-(-)-2-Hydroxyisocaproic acid	1.449	0.002	-0.487
66	Salicylic acid	0.975	0.038	-0.792
67	2-Phenylglycine	0.943	0.045	-0.829
68	Succinylacetone	0.973	0.038	0.405
69	Veratric acid	0.944	0.045	-0.573
70	Acetyl-DL-valine	0.956	0.042	0.264
71	Salicylic acid	1.034	0.027	-0.574
72	Indoxylsulfuric acid	1.573	0.001	0.542
73	2-Isopropylmalic acid	0.934	0.047	-0.817
74	Caffeic acid 3-sulfate	0.929	0.048	-2.412
75	Dihydroferulic acid 4-sulfate	1.351	0.003	-1.229
76	Pyridoxal phosphate	0.965	0.040	-1.215
77	2,3-Dihydroxybenzoic acid	1.702	0.000	-1.477
78	L-Glutamine	1.070	0.022	0.288
79	3-Methyladipic acid	1.838	0.000	-1.158
80	Ferulic acid 4-sulfate	1.903	0.000	-1.748
81	Isoferulic acid 3-O-glucuronide	1.438	0.002	-2.353
82	2-Keto-D-gluconic acid	1.594	0.000	-0.890
83	(±)-Propionylcarnitine	1.639	0.000	0.816
84	Indolelactic acid	1.420	0.002	1.305
85	3-Methylsuberic acid	1.460	0.001	-1.358
86	L-Homocitrulline	1.484	0.001	-1.419
87	Androsterone sulfate	1.735	0.000	0.680
88	L-Homotyrosine	1.384	0.003	-0.464

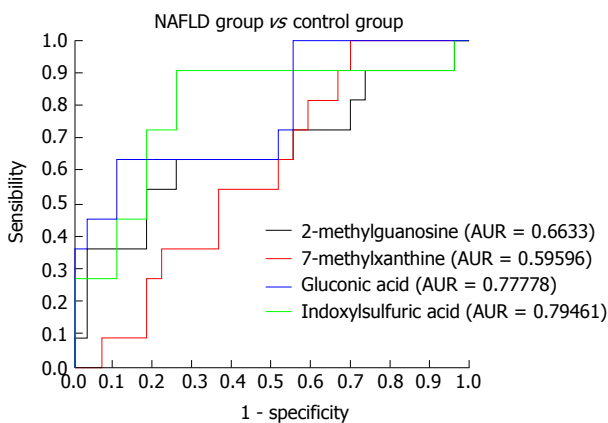


Figure 5 Receiver operating characteristic curves for 2-methylguanosine, 7-methylxanthine, gluconic acid, and indoxylsulfuric acid in the non-alcoholic fatty liver disease and control groups. NAFLD: Non-alcoholic fatty liver disease.

To determine the exact progress of NAFLD, we investigated the metabolic changes involved in NAFLD and NASH. Urinary metabolomics might provide a better understanding of the pathogenesis of NAFLD and reveal key markers that can differentiate between NAFLD from NASH.

In the NAFLD and control groups, the gender ratio showed no difference. While in the NASH group, there were more males than females, which might be because that more females visit their doctor earlier than males, and might not develop NASH, according to the doctor's experience. Age and height were similar in the NAFLD, NASH, and control groups, whereas body weight and BMI were significantly higher in the NASH than in the NAFLD and control groups. These findings suggested a link between obesity and NASH. Parameters of liver function and blood lipids differed in patients with NASH and NAFLD, indicating that

metabolic changes occurred during the progression of NAFLD to NASH. One of the overlapping differentially expressed metabolites, pyroglutamic acid, is involved in glutathione metabolism, a finding consistent with abnormal liver function. Another metabolite, L-carnitine, is involved in bile secretion, perhaps explaining the difference in blood lipid levels between the NAFLD and NASH groups.

Animal experiments have identified metabolic changes in mice with NAFLD or NASH^[18]. For example, the concentrations of triglycerides, cholesterol, and intermediates of the methionine cycle were reported to be altered^[19]. In addition, phospholipid and bile acid metabolism were disrupted^[20] in mouse models of NASH.

Metabolic changes have also been detected in clinical trials. Serum glucose, lactate, glutamate/glutamine, and taurine concentrations were reported to differ between patients with NAFLD and healthy controls^[21]. Bile acids and markers of glutathione, lipid, and amino acid metabolism were also observed to differ between NAFLD patients and controls^[22]. The present study found differences in metabolites of amino acids and nucleic acids in NAFLD patients and controls, with the concentration of hypoxanthine being especially lower in patients with NAFLD. NAFLD is characterized by disorders in hypoxanthine and xanthine metabolism, which lead to lipid peroxidation and oxidative stress, producing increased amounts of free radicals^[23]. Hypoxanthine and xanthine concentrations can be used to estimate the degree of injury to hepatocytes^[24].

This study also showed that the concentration of carnitine in urine was much lower in NAFLD patients than in the healthy controls. Carnitine not only supplies energy for the oxidation of fatty acids^[25], but also eliminates free radicals that can destabilize cell

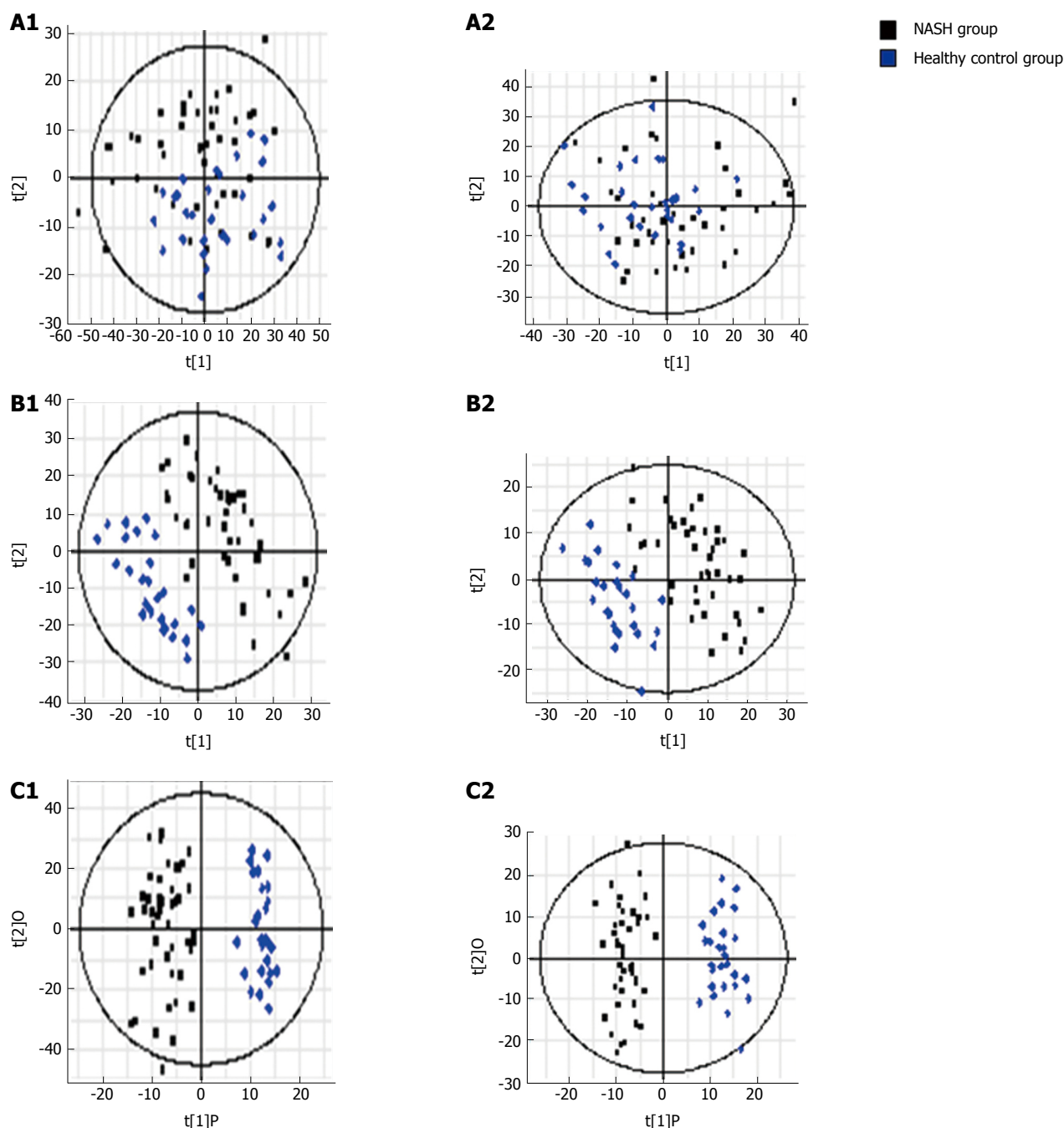


Figure 6 S-plots following (A) PCA, (B) PLS, and (C) OPLS analyses with (A1, B1, C1) electrospray ionization (ESI+) and without (A2, B2, C2) electrospray ionization (ESI-) in the non-alcoholic steatohepatitis and control groups. PCA: Principal component analysis; NASH: Non-alcoholic steatohepatitis.

membranes^[26]. Low carnitine concentrations can result in cell oxidative damage, and fatty acid synthesis and energy metabolism disorders^[27-29], ultimately resulting in NAFLD.

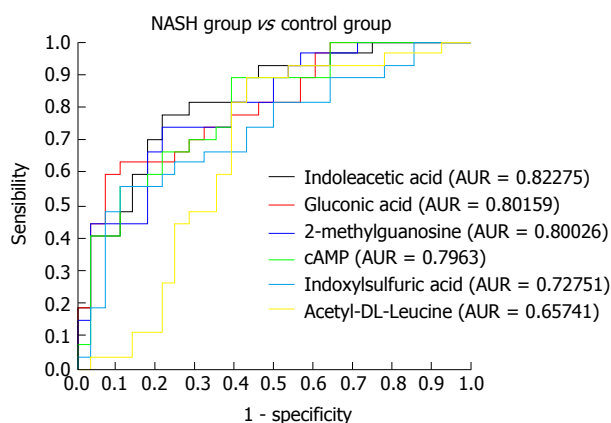
The concentrations of amino acids and their metabolic intermediates were generally higher in patients with NASH patients than in healthy individuals. Most amino acids are synthesized and degraded in the liver; thus, injury to the liver can result in abnormalities in the metabolism of amino acids and the release of amino acids from hepatocytes^[30]. Thus, amino acid levels will be higher in the urine of NASH

patients than in healthy controls. We also found that cortisol concentrations were significantly lower in the urine of NASH patients compared with that in the controls, indicating possible neuroendocrine changes in NASH patients. Cortisol concentrations have been reported to correlate with the severity of NAFLD^[31].

Comparisons between the groups of patients with NAFLD and NASH showed that most of the differentially expressed metabolites were nucleic acids and amino acids. The level of cholinesterase was significantly lower in patients with NASH than with NAFLD. Low levels of cholinesterase will have negative effects on the

Table 5 Urinary metabolites differentially expressed in patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

No.	Metabolites	VIP-value (OPLS-DA)	P value (t-test)	Fold change
ESI+				
1	L-Carnitine	1.253	0.067	-0.727
2	L-Dopa	0.952	0.092	0.234
3	Acetylcarnitine	1.181	0.094	-0.698
4	L-Histidine	1.229	0.081	-1.339
5	Pyroglutamic acid	1.541	0.021	0.143
6	3-Methylxanthine	2.403	0.002	-1.981
7	α -D-Glucose	1.739	0.013	-1.376
8	5-Hydroxyferulate	0.895	0.058	-0.266
9	2-Oxosuberate	0.850	0.073	-0.315
10	p-Hydroxyphenylacetic acid	1.354	0.095	0.398
11	3-Indoleacetic Acid	0.915	0.052	-0.378
12	β -Estradiol	1.883	0.002	-0.635
13	Phosphorylcholine	1.731	0.032	0.716
14	17 α -Hydroxypregnenolone	0.854	0.087	-0.281
15	Deoxycorticosterone	0.865	0.093	-0.312
16	Progesterone	0.866	0.084	-0.301
ESI-				
17	2-Keto-glutaramic acid	1.653	0.027	0.210
18	cAMP	1.659	0.041	0.196
19	7-Methylxanthine	1.595	0.036	-0.925
20	(S)-(-)-2-Hydroxyisocaproic acid	1.344	0.089	-0.250
21	Gluconic acid	1.638	0.025	-1.111
22	N-Acetylproline	1.865	0.021	0.399
23	Acetyl-DL-valine	1.636	0.053	0.284
24	Pyridoxal phosphate	1.490	0.050	-0.990
25	N-Acetyl-DL-tryptophan	1.440	0.059	-0.951
26	2-Keto-D-gluconic acid	1.258	0.088	-0.340
27	D-(+)-3-Phenyllactic acid	2.021	0.017	-1.621
28	Indoleacetic acid	1.495	0.051	0.832
29	3-Hydroxy-sebacic acid	1.270	0.092	-0.292
30	Sebacic acid	1.847	0.024	0.420
31	Deoxyguanosine	1.548		

**Figure 7** Receiver operating characteristic curves for indoleacetic acid, gluconic acid, 2-methylguanosine, cAMP, indoxylsulfuric acid, and acetyl-DL-leucine in the non-alcoholic steatohepatitis and control groups. NASH: Non-alcoholic steatohepatitis.

synthesis and secretion of very low-density lipoprotein (VLDL). This can result in an inability to transport TG out of hepatocytes, which can result in liver steatosis^[32,33]. Deposits of excess fat can cause lipid peroxidation and damage to the antioxidant barrier^[25,26], an important step by which NASH develops from NAFLD^[34]. In-

terestingly, we also found that the level of indoleacetic acid was much higher in the NASH group compared with that in the NAFLD group. This was consistent with findings showing that the indoleacetic acid concentration correlates with liver damage^[35].

The alterations observed in the NAFLD and NASH groups mainly affect energy^[19]. Differential levels of hormones, cytokines, and neurotransmitters may result in abnormal energy metabolism in patients with NAFLD^[36], which is consistent with our results. Alterations in hepatic mitochondrial function in NAFLD patients might influence lipid metabolism and promote oxidative stress^[37], eventually resulting in changes in metabolites. Pathway analysis of the overlapping metabolites indicated that amino acid metabolism and pentose phosphate pathways might be involved in the progression of NAFLD to NASH. Alterations in amino acid metabolites represent adaptive physiological responses to hepatic stress in patients with NASH^[38]. Glycometabolism, including the pentose phosphate pathway, might be altered, inasmuch as insulin resistance is one of the primary causes of NAFLD^[39]. Many of these compounds might be associated with biochemical perturbations associated with liver dysfunction and inflammation^[40]. The alterations in metabolomics we

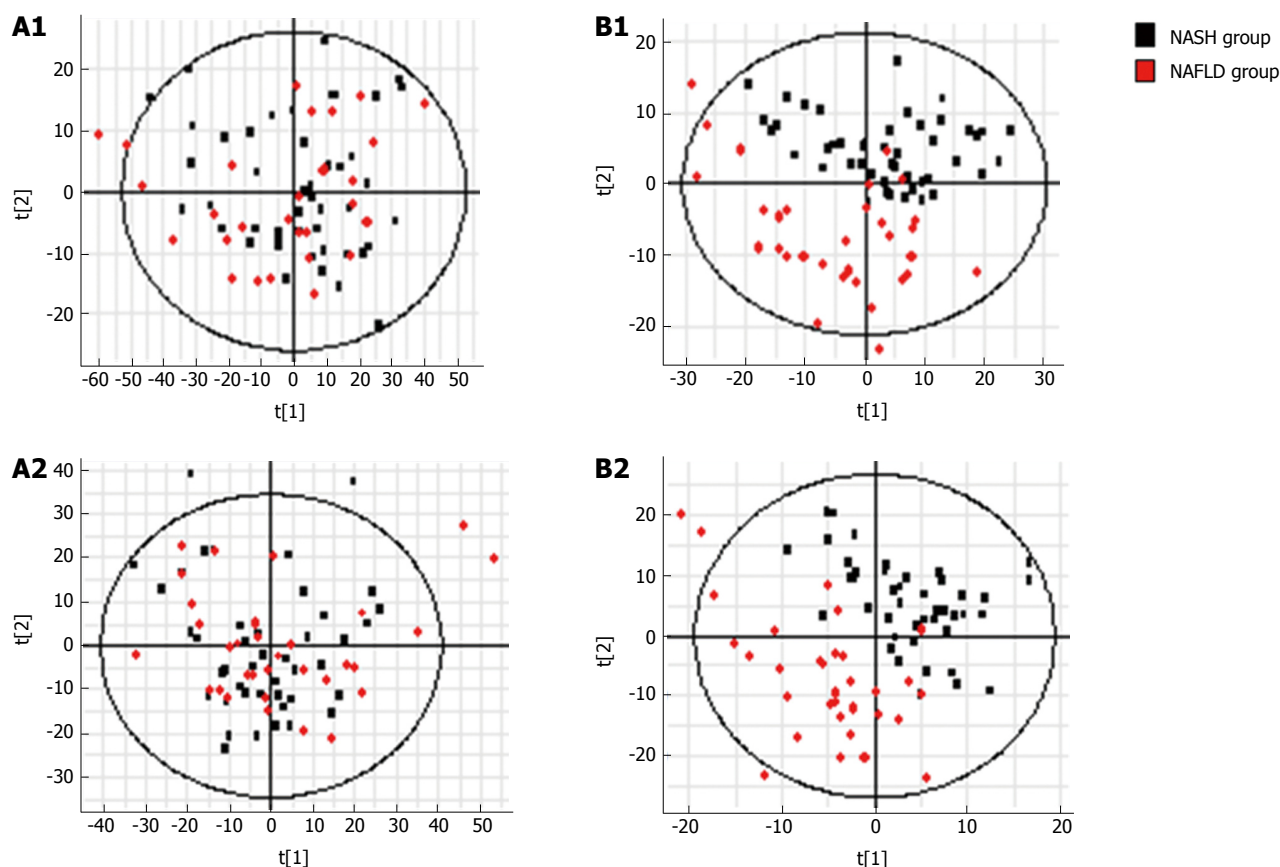


Figure 8 S-plots following (A) PCA and (B) PLS analyses with (A1, B1) electrospray ionization (ESI+) and without (A2, B2) electrospray ionization (ESI-) in the non-alcoholic steatohepatitis and non-alcoholic fatty liver disease groups. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

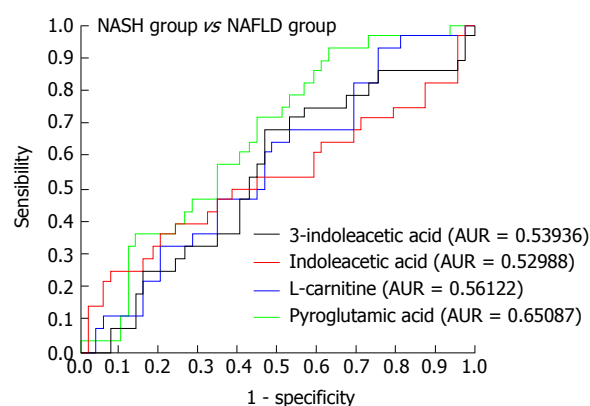


Figure 9 Receiver operating characteristic curves for 3-indoleacetic acid, indoleacetic acid, L-carnitine, and pyroglutamic acid in the non-alcoholic steatohepatitis and non-alcoholic fatty liver disease groups. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

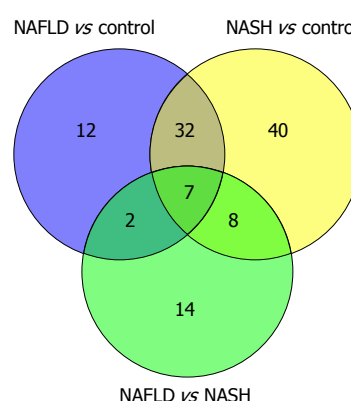


Figure 10 Venn diagram of metabolites differentially expressed in urinary samples of the non-alcoholic fatty liver disease vs control, non-alcoholic steatohepatitis vs control, and non-alcoholic fatty liver disease vs non-alcoholic steatohepatitis groups. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

observed were consistent with previously reported changes in biochemical parameters.

Statistical analysis identified a panel of biomarkers involved in energy metabolism, amino acid metabolism, and glycometabolism, which might provide clues to the potential mechanism involved in the progress from NAFLD to NASH. These biomarkers could be used to distinguish between NAFLD from NASH effectively. These biomarkers might be diagnostic for NASH and

could act as indicators of the efficacy of therapeutic interventions.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of pathological conditions, including simple steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis. The prevalence of NAFLD is linked strongly to obesity, insulin

resistance, and a cluster of metabolic disorders, including hypertriglyceridemia and hyperuricemia, which impair health seriously. No standard treatment exists currently to manage NAFLD, or even NASH. Identification of metabolic differences among stages of NAFLD might result in the development of more effective and specific treatments for NAFLD and NASH. This study was designed to investigate correlations between disease stages and urine metabonomics in patients with NAFLD, specifically to determine whether urine metabonomics could be used to distinguish NAFLD from NASH, which would aid the diagnosis and treatment of NAFLD.

Research frontiers

A panel of biomarkers that can distinguish between NAFLD and NASH and can help to determine the molecular mechanism involved in the process of development of NASH from NAFLD was developed. Urinary biomarkers may be diagnostic in these patients and might be used to assess responses to therapeutic interventions.

Innovations and breakthroughs

Metabolic changes have been detected in clinical trials. Serum glucose, lactate, glutamate/glutamine, taurine concentrations, bile acids, markers of glutathione, lipids, and amino acid metabolism have been reported to differ between patients with NAFLD and healthy controls. Among those findings, low carnitine concentrations could result in cell oxidative damage, fatty acid synthesis, and energy metabolism disorders, ultimately resulting in NAFLD. Cortisol concentrations have been reported to correlate with the severity of NAFLD and indole acetic acid concentration correlates with liver damage. Differential levels of hormones, cytokines, and neurotransmitters might result in abnormal energy metabolism in patients with NAFLD.

Applications

The urinary biomarkers found in this study might be diagnostic in these patients and could be used for diagnose and to evaluate the treatment of NAFLD.

Terminology

NAFLD means nonalcoholic fatty liver disease. In our study, patients with NAFLD refers to those patients that were diagnosed using B ultrasound and their liver functions were normal. NASH means nonalcoholic steatohepatitis. In our study, patients with NASH refers to those patients that were diagnosed using B ultrasound and their liver functions were abnormal. Principal component analysis means principal component analysis. Partial least squares discriminant analysis refers to partial least squares-discriminate analysis. Orthogonal projections to latent structures-discriminant analysis is orthogonal projections to latent structures discriminant analysis. All these analyses were used to distinguish different groups of patients or controls.

Peer-review

This article investigated urinary biomarkers to distinguish NAFLD from NASH, which could help to determine the molecular mechanism involved in the process of developing NASH from NAFLD and improve the diagnosis and treatment of NAFLD. Not only the results, but also the methods will be attractive for readers.

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P- Reviewer: Lee HC, Miura K **S- Editor:** Ma YJ
L- Editor: Stewart G **E- Editor:** Zhang FF





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ISSN 1007-9327



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