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***Clinical Trials Study***

**Urinarymetabolomics analysis identifies key biomarkers of different stages of nonalcoholic fatty liver disease**

Dong s *et al*. Biomarkers of stages in NAFLD

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**Abstract**

***AIM***

To find out a panel of biomarkers which can distinguish between non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) and explore molecular mechanism involved in the process of development of NASH from NAFLD.

***METHODS***

Biomarkers may differ during stages of with NAFLD. Urine and blood were obtained from non-diabetic subjects with NAFLD and steatosis, with normal liver function (*n =* 33) and 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 by 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 metabonomics found differences in 31 metabolites in 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 suggest that a panel of biomarkers can distinguish between NAFLD and NASH and can help determine the molecular mechanism involved in the process of development of NASH from NAFLD. Urinary biomarkers may be diagnostic in these patients and may be used to assess responses to therapeutic interventions.

**Key words:** Nonalcoholic fatty liver disease; Steatohepatitis; Urinary metabonomics

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**Core tip:** To find out biomarkers which can distinguish between nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), urine and blood were obtained from NAFLD (*n =* 33), NASH (*n =* 45) and healthy controls (*n =* 30). Urinary metabonomics found differences in 31 metabolites between NAFLD and NASH, including nucleic acids and amino acids. Pathway analysis 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. These biomarkers can distinguish between NAFLD and NASH and can help determine the mechanism involved in the development of NASH from NAFLD.

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**INTRODUCTION**

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of pathologic 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 rate of NAFLD is strongly linked to obesity, insulin resistance and a cluster of metabolic disorders, including hypertriglyceridemia and hyperuricemia[3], that seriously impair health[4].

No standard treatment currently exists for managing NAFLD, oreven 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 stages of NAFLD may result in the development of more effective and specific treatments for NAFLD and NASH.

Urine metabonomics[9] is a good method of assessing metabolic differences among different stages of NAFLD. Although urinary metabolomics data have been obtained in patients with NAFLD, NASH and liver cirrhosis[10], few studies to date have used this method to compare patterns in patients at different stages of NAFLD. This study was designed to investigate correlations between disease stages and urine metabonomics in patients with NAFLD, specifically to determine whether urine metabonomics can be used todistinguish 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 fromJanuary 2013 to May 2014. Healthy volunteer controls were enrolled from 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. Results of laboratory tests and ultrasound were also recorded. Urine samples for metabolic profile 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 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 ALT activity or other liver function parameters.

***Inclusion criteria***

Both 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 test, NAFLD patients weredivided 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 centrifuged at 4 °C for 15 min at 1509.3×g. The supernatantswere frozen and stored at −80 °C until analysis. If required, urine samples were transported using Drikold.

***LC/MS analysis***

Pretreatment: 100 ul urine and 300 ul acetonitrile were vortexed for 3 min and then centrifuged at 12000 r/min, 4 ℃ for 10 min. Supernate were kept as prepared samples for further performance.

LC separation was performed on an Agilent 1200 series LC system. Aliquots of 2 µl of 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 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 standardswere 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 online database (http://metlin.scripps.edu/), and addition, presently 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 profileson LC/MS were determined by principal component analysis and partial least squares discriminant analysis.

To validate the importance of metabolites, and to furthergauge theirability todistinguish among patients with NASHand NAFLD and healthy controls, their potential predictive utility for the process of NAFLDwas assessed by ROC curve analysis. ROC analysis was performedusingMS peak areas corresponding to metaboliteconcentrations in each of the three subjects groups. Areas under the ROC curve were calculated using RORC package.

***Quality control***

The actual measurements from each patient’s laboratory 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 difference among the three groups in patient number, age and height. Weight and BMI were significantly higher in the NAFLD and NASH groups than in the control group (Table 1).

Compared with 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), alanine aminotransferase (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 NASH group ultrasound examinations(Table 2, Figure 2).

***Urine metabonomics***

Principal component analysis (PCA) was performed using samples from the three groups of subjects. S-plots showed obvious metabolic differences among these three groups (Figure 3).

**NAFLD group *vs* control group:** This was followed by pairwide comparisons. Urinary metabonomics were used to assess differences between the NAFLD and control groups. Principal component analysis (PCA) showed a spectral separation between these two groups, indicating significant metabolic differences.This was further supported by PLS-DA and OPLS-DA(Figure 4). After filtering out interference signals, 53 different metabolites were detected;mainly nucleic acids and amino acids (Table 3). Concentrations of the nucleic acid metabolites hypoxanthine, xanthine and carnitine were lower in the urine of patients with NAFLD than in control subjects. In addition, 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 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 metabonomics were also used to assess differences between the NASH and control groups. Analysis by PCA 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 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 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 NAFLDand NASHshowed spectral separation between the two groups of samples, indicating significant metabolic differences. PLS-DA was performedto 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 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 (Figure 9).

**Key differential metabolites among the NALFD, NASH, and control groups:** The differentially expressedmetabolites in the three pairwise comparisons were combined to determine themetabolitesthat overlapped in the three groups. Seven metabolites were screened through Venn analysis: L-carnitine, acetylcarnitine, gluconic acid, deoxycorticosterone, 2-keto-D-gluconic acid, pyroglutamic acid, and indolelactic acid (Figure 10). KEGG 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, maybe closely related to the pathological processes of NAFLD and NASH.

# DISCUSSION

Obesity, insulin resistance and associated metabolic perturbations are frequently observed in patients with NAFLD[14,15]. NASH is a type of NAFLD with serious abnormalities in liver function[16]. NAFLD has a great impact on health, affecting many bodilysystems[17]. To determine the exact progress of NAFLD, we investigated metabolic changes involved in NAFLD and NASH.Urinary metabolomicsmay provide better understanding of the pathogenesis of NAFLD and may reveal key markers that can differentiate between NAFLD from NASH.

In NAFLD and the control group, the gender ratio has no difference. While in NASH group, there were more male than female which may due to that more female came to doctor earlier than male so they would not develop to NASH according to the doctor's experience. Age and heightwere 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 suggest a link betweenobesity andNASH.Parameters of liver function and blood lipidsdiffered in patientswith NASH and NAFLD, indicating that metabolic changes occurred during the progression of NAFLD to NASH.One of the overlappingdifferentiallyexpressed 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 levelsbetween the NAFLD and NASH groups.

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

Metabolic changes have also been detected in clinical trials. Serum glucose, lactate, glutamate/glutamine, and taurine concentrations have been reported to differ in patients with NAFLD and healthy controls[21]. Bile acids and markers of glutathione, lipid and amino acid metabolism differ in NAFLD patients and controls[22]. This 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 disordersin hypoxanthine and xanthinemetabolism, with these aberrations leadingto lipid peroxidation and oxidative stress, producing increased amounts of free radicals[23]. Hypoxanthine and xanthine concentrations can be used to estimate the degree of injuryto hepatocytes[24].

This study also showed that the concentration of carnitine in urine was much lower in NAFLD patients than in healthy controls. Carnitine not only supplies energy for the oxidation of fatty acids[25] but eliminates free radicals that can destabilizecell membranes[26]. Low carnitineconcentrations can result in cell oxidative damage, 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 patientsthan 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 levelswill 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 than in controls, indicatingpossible neuroendocrine changes in NASH patients. Cortisol concentrations have been reported to correlate with the severity of NAFLD[31].

Comparisons in the groups of patients with NAFLD and NASH showed that most ofthe differentially expressed metabolites were nucleic acids and amino acids. The level of cholinesterase was significantly lower in patients with NASH than with NAFLD. A low content of cholinesterase will have negative effects on the synthesis and secretion of very low-density lipoprotein (VLDL). This can result in the inability to transport triglycerides (TG) out of hepatocytes, which can result in liver steatosis[32,33]. Deposits of excess fatcan cause lipid peroxidation and damage to the antioxidant barrier[25,26], an important step by which NASH develops from NAFLD[34]. Interestingly, we also found that the level of indoleacetic acid was much higher in the NASH than in the NAFLD group. This was consistent with findings showing that 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], findings consistent with our results. Alterations in hepatic mitochondrial function in NAFLD patients may influence lipid metabolism and promote oxidative stress[37], and may eventually result in changes in metabolites. Pathway analysis of the overlapping metabolites indicated thatamino acid metabolism andpentose phosphate pathways may 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, may be altered, inasmuch asinsulin resistance is one of the primary causes of NAFLD[39]. Many of these compoundsmay be associated with biochemical perturbations associated with liver dysfunctionand inflammation[40]. The alterations in metabonomics we 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 that may provide clues to the potential mechanism involved in the progress from NAFLD to NASH.These biomarkers may be used to effectively distinguish between NAFLD from NASH. These biomarkers may be diagnostic for NASH and may act as indicators of the efficacy of therapeutic interventions.

**comments**

***Background***

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of pathologic conditions, including simple steatosis, nonalcoholic steatohepatitis (NASH) and cirrhosis. The rate of NAFLD is strongly linked to obesity, insulin resistance and a cluster of metabolic disorders, including hypertriglyceridemia and hyperuricemia, that seriously impair health. No standard treatment currently exists for managing NAFLD, or even NASH. Identification of metabolic differences among stages of NAFLD may 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 can be used to distinguish NAFLD from NASH, which would shed a light in dignosis and treatment of NAFLD.

***Research frontiers***

A panel of biomarkers can distinguish between NAFLD and NASH and can help determine the molecular mechanism involved in the process of development of NASH from NAFLD. Urinary biomarkers may be diagnostic in these patients and may 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, lipid and amino acid metabolism have been reported to differ in patients with NAFLD and healthy controls. Among those findings, low carnitine concentrations can 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; Indoleacetic acid concentration correlates with liver damage; Differential levels of hormones, cytokines, and neurotransmitters may result in abnormal energy metabolism in patients with NAFLD.

***Applications***

Urinary biomarkers found in this study may be diagnostic in these patients and may be used to for diagnosis and evaluate the treatment of NAFLD.

***Terminology***

NAFLD, Nonalcoholic fatty liver disease. In our study, patients with NAFLD mean those patients which were dignosed with B ultrasound and their liver function were normal; NASH, nonalcoholic steatohepatitis. In our study, patients with NASH mean those patients which were dignosed with B ultrasound and their liver function were abnormal; PCA, Principal component analysis. PLS-DA, partial least squares-discriminate analysis. OPLS-DA, orthogonal projections to latent structures discriminant analysis. All these analysis were used to distinguish different groups.

***Peer-review***

This article investigated the urinary biomarkers to distinguish NAFLD from NASH which can help determine the molecular mechanism involved in the process of development of NASH from NAFLD and shed a light in diagnosis and treatment for NAFLD. Not only the results but also the methods will be attractive for readers.

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**Table 1 Demographic and clinical characteristics of study participants (mean ± SD)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Group**  | **NASH** | **NAFLD** | **Healthy group** |
| Number  | 45 | 33 | 30 |
| Age (yr) | 39 ± 10 | 44 ± 14 | 39 ± 4 |
| Gender  | Male(38)female(7) | Male(19)female(14) | Male(21)female(9) |
| Height (cm) | 169 ± 8 | 170 ± 6 | 168 ± 6 |
| Weight (kg) | 76 ± 13b | 78 ± 11b | 68 ± 9 |
| BMI (kg/m2) | 26.40 ± 3.49b | 26.81 ± 3.43b | 23.73 ± 1.95 |

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

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

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

**NAFLD patients**

**Collection of clinical information**

**NASH**

**(patients**

**withabnormal transaminase)**

**(*n* = 45)**

**NAFLD**

**(patients**

**with normal transaminase)（*n* = 33）**

**Collection of basic information collection**

**Healthy**

**Controls**

**(*n* = 30)**

**Urine**

**samples**

**Ultrasound**

**Laboratory**

**results**

**Metabonomics**

**analysis**

**Illustrate the differences in metabonomics between NAFLD and NASH patients**

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



A



B



c



D



healthy control group

NASH group

NAFLD group

NASH group

E

NASH group

NASH group

**Figure 2** **Characteristics of 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 alfa-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 NASH group 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, dP < 0.01 *vs* the NAFLD group.

 

A B

Figure 3 S-plots of PCA analysis (a) with electrospray ionization (ESI+) and (b) without electrospray ionization (ESI-) in the NASH, NAFLD and control groups. PCA: Principal component analysis; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

 

A1 B1 C1



A2 B2 C2

**Figure 4 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 NAFLD and control groups.** PCA: Principal component analysis; NASH: Non-alcoholic steatohepatitis; NAFLD: Non-alcoholic fatty liver disease.

**Table 3 List of urinary metabolites differentially expressed in NAFLD 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 | Nα-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 | 6β-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  |



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

 

A1 B1 C1



A2 B2 C2

**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 NASH and control groups.** PCA: Principal component analysis; NASH: Non-alcoholic steatohepatitis.

**Table 4 List of urinary metabolites differentially expressed in NASH patients and healthy controls**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **No.** | **Metabolites** | **VIP-value****(OPLS-DA)** | ***P*-value****(*t*-test)** | **Fold change** |
| E |  |  |  |  |
| 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 | Nα-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 | 6β-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-aminosalicyluric 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 | Salicyluric 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 7 ROC curves for indoleacetic acid, gluconic acid, 2-methylguanosine, cAMP, indoxylsulfuric acid, and acetyl-DL-leucine in the NASH and control groups.** NASH: Non-alcoholic steatohepatitis.

 

A1 B1



A2 B2

**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 NASH and NAFLD groups.** NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

**Table 5 List of urinary metabolites differentially expressed in patients with NAFLD and NASH**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **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 | 17α-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 | Indoleactic 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 9 ROC curves for 3-indoleacetic acid, indoleacetic acid, L-carnitine, and pyroglutamic acid in the NASH and NAFLD groups.** NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.



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