Potential New Biomarkers Discovery of Early Nonalcoholic Fatty Liver Disease in Human by Liquid Chromatography–Mass Spectrometry

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Research

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Abstract

Background

NAFLD is a common metabolic disorders disease which influenced 20~30% people. NAFLD can progress to cirrhosis, liver fibrosis and even liver cancer. Liver puncture is the gold standard. However, due to its trauma and possible complications, its clinical use is currently limited. Therefore, it is of great clinical significance and value to find a noninvasive biochemical index that can diagnose NAFLD early.

Objective

Our aim was to identify the potential biomarkers in NAFLD people in early stage via untargeted metabolomics study.

Methods

In our research, From January to October 2019, 224 patients aged 18-55 were selected from the outpatient department and ward of gastroenterology department of Putuo Hospital in Shanghai.According to the NAFLD diagnostic criteria of the guidelines for diagnosis and treatment of nonalcoholic fatty liver disease (2018) formulated by the National workship on Fatty liver and alcoholic liver disease and Chinese Society on Hepatology, they were divided into the healthy control group and the experimental group.Besides,on the same day, the height, weight, waist, BMI, blood pressure and heart rate of patients were measured, and fasting blood was taken to obtain blood glucose,ALT, AST, TB, DB, TP, ALB, Che, ALP, γ - GT, TG, TC, HDL-C, LDL-C and other serum data.Serum samples were analyzed using LC/MS and data was processed by SIEVE software and simca-P to validate the potential biomarkers. The altered metabolites were identified by variable importance in projection value (VIP > 1) and ANOVA (p<0.01). The pathway analysis was performed by using MetaboAnalyst 4.0. In addition, our project has passed the review of ethics committee of Putuo Hospital Affiliated to Shanghai University of traditional Chinese medicine, and its ethics approval number is ptec-a-2018-49-1.

Results

The serum biochemical indicators of early NAFLD patients showed no significant difference with NC ($p>0.05$). While there was significant difference of blood lipids indicators between NAFLD and NC ($p<0.001$). Finally, 55 metabolites were identified and the AUC of ROC curve results showed that new identified biomarkers owned high predictability and reliability.

Conclusion

It is found that 15 metabolites in serum were of great diagnostic value in early NAFLD patients. AUC of these biomarkers (>0.9) were much higher than clinical indicators (0.770). This may be worth further research in the clinic.

Background

In recent years, the trend of liver diseases is changing from traditional infectious diseases to metabolic disorders[1]. In general, liver disease is a high incidence disease in Asia, because there are many complicated factors leading to liver disease in Asia [2–4]. Besides, nonalcoholic fatty liver disease (NAFLD) is also a common liver disease in developed country [2, 5] NAFLD is the extension of obesity and its associated metabolic disorders, which influenced 20 ~ 30% people in the world. More than 20–30% of patients with NAFLD develop progressive liver disease with steatohepatitis and fibrosis that is able to lead in cirrhosis in 10–20% of cases[6] .NAFLD is the fastest developing reason of cirrhosis in the China—a concerning trend given the possible association between NAFLD-related cirrhosis and hepatocellular cancer (HCC)[7]. HCC has also been found to rise in patients with NAFLD in the absence of cirrhosis[8].

The major defects of NAFLD assessment is lacking accurate non-intrusion diagnosis method, especially in early stage. The gold index of NAFLD is liver biopsy, however, due to its invasive, unavoidable sampling error, poor short-term repeatability, subjective differences among observers and other factors, liver biopsy is difficult to carry out widely as a routine examination method in clinical practice, nor should it be repeated in a short period of time, which is extremely unfavorable to the follow-up and monitoring of the disease[9]. The most convenient and promising monitor method for NAFLD is still the unusual metabolic biomarkers in blood[10–11].

The most commonly used blood biomarkers currently still were alanine aminotransferase (ALT), aspartate aminotransferase (AST) and so on. These biomarkers will not raise until histological injury of liver occurred[12]. Therefore, the discovery and exploration of new biomarkers at early stage for NAFLD is of crucial significance. Metabolomics, as a high-throughput technology, has been proved that thousands of serum metabolites can be measured and identified simultaneously[13–15], which is suitable for discovery of new biomarkers[16–17]. In our research, the metabolic characteristics of NAFLD blood samples were analyzed to find more meaningful specific biomarkers for NAFLD.

Materials And Methods

Reagents and instrument

Fenclonine were purchased from Aladdin (Shanghai); Methanol and acetonitrile (HPLC grade) were purchased from Fisher Chemicals (Waltham USA); Formic acid was purchased from Merck (Germany); LC-MS system (Ultimate 3000LC, Orbitrap Elite Thremo).

Participants

Subjects: 224 subjects from the outpatient department and ward of digestive department of Putuo Hospital Affiliated to Shanghai University of traditional Chinese Medicine.

Inclusion criteria

There are 112 patients belonged to the NAFLD diagnostic criteria of the guidelines for diagnosis and treatment of nonalcoholic fatty liver disease (2018) [18]formulated by the National workship on Fatty liver

and alcoholic liver disease and Chinese Society on Hepatology.All patients in the group's liver function were within the normal range, and three doctors with the title of deputy director diagnosed the abdominal B-ultrasound of the group as mild fatty liver at the same time, as long as one person thought it was non mild fatty liver, the patient was excluded.The control group consists of outpatient health follow-up population, all patients in this group without any abnormal examination, and no underlying disease. All patients in both groups signed an informed consent form, and were able to successfully complete the collection of medical history, biochemical routine and abdominal B-ultrasonic examination. Moreover, our project has passed the examination of the ethics committee of Putuo District Central Hospital (Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine), and the ethics approval number is PTEC-A-2018-49-1.

Exclusiong criteria

Those with any of the following liver diseases : viral hepatitis, cirrhosis, liver cancer, autoimmune liver disease, alcoholic liver disease, hereditary liver disease, etc ; with a history of excessive alcohol drinking, the amount of alcohol in men≥140g/week; in women≥70g/week; Have taken liver-protective drugs, lipid-lowering drugs or drugs that can cause liver fat deposition; patients who have been diagnosed with diabetes and who have received or are undergoing any hypoglycemic drugs or insulin treatment;patients with severe heart disease (patients with myocardial infarction,heart failure and / or severe arrhythmia); patients with severe infections and severe trauma; women who are pregnant or likely to be pregnant and breastfeeding; those who cannot comply due to mental illness;those who do not sign the informed consent; patients with thyroid diseases, including hyperthyroidism and hypothyroidism.

Measurements

The medical history and physical examination of the patients were collected by the full-time doctors in the outpatient or ward of the department of gastroenterology, and the general conditions of the patients were recorded in detail, including name,gender, age, waist circumference, smoking history, drinking history, etc.

The height, weight and blood pressure were measured in the morning of the next day. The anthropometric parameters were measured by a specially assigned person to calculate the body mass index, body height and waist to hip ratio. During weight measurement, take off shoes, take off coat and wear single underwear. The weight is corrected to kilogram and the height is measured to centimeter. The waist circumference was measured at the middle point of the line between the lower edge of the arch and the iliac spine, accurate to cm; the average value was measured twice. Blood pressure was measured in quiet state for times, 10 minutes apart each time, and the average value was taken for three times.

Laboratory analysis

After fasting for 12 hours overnight, fasting blood was collected from the veins early in the morning the next day and sent to biochemical laboratory of our hospital for blood glucose,ALT, AST, TB, DB, TP, ALB, Che, ALP, γ - GT, TG, TC, HDL-C, LDL-C and other serum data.

Metabolomic analysis

Sample preparation for metabolomics

Serum samples were prepared as follows: 100μl serum was mixed with 400 μl methanol and 5 μl 2-Chloro-DL-phenylalanine (0.3 g/l, internal standard) for extraction. Then the supernatant of each sample was used for analysis after being centrifuged at 15000 r/min at 4℃ for 10 min.

UPLC-Q-Orbitrap-MS analysis conditions

A LC/MS equipment consisting of a UPLC system and Q-Orbitrap Elite spectrometer was used for metabolic analysis. The mobile phase consists of a mixture of water with 0.1% formic acid (A) and Acetonitrile (B). Samples was eluted through a ACQUITY UPLC column (Hss T3, 100mm×2.1 mm, 1.8μm) at a flow rate of 0.3 ml/min for 20 mins with following elution conditions: 0-2 min, 95%A; 2-12 min, 5%A; 12-15 min, 5%A; 15-20 min, 95%A. The mass setting parameters were as follows: (1)heater temperature(300°C); (2)sheath gas flow(45psi); (3)auxiliary gas flow(5L/min); (4)tail gas flow(0.3L/min); (5)electrospray voltage (3.0kV for positive mode and 3.2kV for negative mode); (6) capillary temperature: 350 C; (7) S-LensF Level, (30% for positive mode and 60% for negative mode).

Metabolites identification and pathway analysis

Discriminant metabolic features were identified based on their accurate masses and/or product ion spectra in both negative and positive mode. HMDB, KEGG, mzCloud were searched to assist metabolite identification. The collected MS data were pre-processed by SIEVE software for identification (Thremo Company) and arranged into two-dimensional data matrix in Excel 2013. Then PCA and OPLS-DA were performed by SMICA-P 14.0 software and the variable importance in projection value (VIP) was used to screen differential metabolites. The differential metabolomics pathway analysis was performed by using MetaboAnalyst 4.0.

Statistical analysis

SPSS 21.0 software was used to carry out two-way ANOVA, receiver operating characteristic curve (ROC) and logistic regression results among groups, and the measurement data was expressed as means ± SD. The difference was statistically significant with $p < 0.05$.

Results

Demographic characteristics

From Table 1, we found that among the 224 subjects, the experimental group had significant differences in body weight, BMI, and waist statistics compared with the normal control group ($p<0.001$), while there were no obvious differences in age, gender, height, systolic blood pressure, diastole blood pressure, smoking and drinking($p>0.05$).

Gender

Among the 224 subjects, there were 130 males and 92 females, including 68 males and 44 females in the experimental group, 62 males and 48 females in the normal control group.

Age

In the experimental group, the minimum age was 18, the maximum age was 60, and the average age was 46. The normal control group had a minimum age of 25, a maximum age of 65, and an average age of 41. There was no visible statistical difference between the two groups (p>0.05)

Height

The tallest patient in the experimental group was 196 cm, the shortest patient was 154 cm, and the average height was 169.42 cm; the tallest patient in the normal control group was 183 cm, the shortest patient was 158 cm, and the average patient height was 170.59 cm. There was no significant statistical difference between the two groups (p>0.05)

Weight

Among the 112 patients in the experimental group, 20 were obese, with the heaviest patient weighing 100kg. The average weight of the experimental group was 77.5kg, while that of the normal control group was 62.37kg. The weight of the experimental group was significantly higher than that of the normal control group, with a statistically significant difference (p< 0.001).

BMI

There were six subjects whose BMI were in the normal range□eleven were in the obesity range□ten were in the overweight range; The BMI in the normal control group was within the normal range. The BMI in the experimental group was significantly higher than the normal control group, and the two groups had statistically visible differences (p <0.001).

Blood Pressure

The highest systolic blood pressure in the experimental group was 140mmHg, the lowest was 110mmHg, the highest diastolic blood pressure was 85mmHg, and the lowest was 70mmHg; the normal control group had the highest systolic blood pressure of 135mmHg, the lowest was 125mmHg, the highest diastolic blood pressure was 88mmHg, and the lowest was 65mmHg. There was no clear statistical difference between the two groups (p>0.05)

Waist

The maximum waist circumference of the experimental group was 116 cm and the shortest was 76 cm; the maximum waist circumference of the normal control group was 95 cm and the shortest was 70 cm. The waist circumference of the experimental group was significantly higher than that of the normal control group, and there was significant difference between the two groups (p<0.001).

Smoking

Among 224 subjects, we found that 145 subjects had a history of smoking, of which 62 in the experimental group accounted for 55.4% of the experimental group; the normal control group accounted for 83, accounting for more than 74% of the normal control group. There was no obvious statistical difference between the two groups ($p>0.05$)

Drinking

We found that a total of 145 patients had a history of drinking, of which 76 were in the experimental group, accounting for more than 67% of the experimental group; 69 were in the control group, accounting for more than 62% of the normal control group. There was no significant statistical difference between the two groups ($p>0.05$).

Serum index

From Table one, we found that there was no remarkable difference in biochemical indexes (included TBA, CHE, TB, DB, TP, ALB, γ-GT, ALP, AST, ALT and GLU) between the experimental group and the normal group ($p>0.05$). However, there were conspicuous differences in all blood lipid indexes (HDL, LDL, TC and TG) between the two groups. ($p<0.001$, $p<0.01$, $p<0.01$, $p<0.01$).

The subjects in this experimental group were diagnosed with early NAFLD. Because these patients are unwilling to cooperate with liver puncture testing, we selected patients with abdominal fatty ultrasound that indicates mild fatty liver and normal liver function. There was no significant statistical difference between the two groups (Supplement Fig. 1).Among 112 NAFLD patients in the experimental group, 94 had TG higher than 1.69mmol/l, 88 had TC higher than 5.2mmol/l, 92 had LDL-C higher than 3.37mmol/l, and 95 had HDL-C lower than 1.04mmol/l. It is worth mentioning that over 70& NAFLD patients had abnormal in TG, TC, HDL-C in the experimental group. There were statistically noteworthy differences in lipid levels between the two groups.

PCA of serum samples in NAFLDs

The pre-processed MS data in excel were analyzed in Simca-P 14.0 software. The PCA was performed in positive and negative ionization, respectively. Quality control samples were determined for instrument precision and results showed the stability of UPLC-MS/MS system. NAFLDs and normal groups were separated completely in PCA score plots **(Fig.1)**. The cumulative values of R^2X and Q^2Y in both modes showed the high discriminative and predictive degree of analytical mode.

OPLS-DA and Metabolites identification of serum in NAFLDs

OPLS-DA was employed on NAFLD and Normal groups to identify the potential metabolic biomarkers. The R^2Y and Q^2Y values of were 0.986 and 0.895 respectively in positive mode and 0.957 and 0.877 in negative mode. It indicated that the discriminative and predictive degree of methods meets the analysis requirement. Then variable importance in projection (VIP) and p-value were both used to search potential metabolic

biomarkers. 55 metabolites in serum met the retrieval requirements (*VIP* > 1 and $p < 0.01$) and were identified by searching in the library **(Table.2)**.

Pathway enrichment analysis

Pathway enrichment was achieved on MetaboAnalyst 4.0 and results were showed in **Fig.2.** Pathways of significant difference ($p < 0.05$) were as follows: (1) Phenylalanine metabolism; (2) Aminoacyl-tRNA biosynthesis; (3) Glycerophospholipid metabolism; (4) Ether lipid metabolism; (5) Fatty acid biosynthesis; (6) Citrate cycle (TCA cycle).

Diagnostic accuracy analysis for identified biomarkers for early NAFLD patients.

A detailed summary of AUC, 95%CI lower and upper limit, sensitivity and specificity for identified serum metabolites were showed in **Table.2.** AUC of 15 metabolites in serum were over 0.9, which showed high diagnostic values. The ROC curve lipids four (TC, TG, HDL and LDL) and blood biochemistry (ALT, AST, ALP and γ-GT) was made to compare with the new biomarkers. The predictive value of individual indicator was no more than 0.770 (Fig.4).

Discussion

NAFLD has become the most common chronic liver disease in China [19-20]. It is a liver condition characterized by insulin resistance, hepatic steatosis and frequently prediabetes or Type 2 diabetes mellitus (T2DM). The relationship between Type 2 diabetes mellitus and NAFLD has been fully confirmed, and insulin resistance is not only the basis of Type 2 diabetes mellitus, but also an important mechanism of NAFLD[21], The study found that the risk of NAFLD was increased in Type 2 diabetes mellitus, while NAFLD can also be an independent risk factor for the progression of diabetes mellitus[22-23], thus forming a vicious circle.The specific prevalence of NAFLD is still unknown, depending on the method used for screening, which is lower when liver transaminase and/or liver ultrasound are used, and higher with gold standard magnetic resonance spectroscopy (MRS). The prevalence of NAFLD in industrialized countries is believed to be between 40% and 50%, and even higher in patients with T2DM, with a prevalence of up to 90% in morbidly obese patients [24-26]. About 40% of people with NAFLD are believed to be likely to go on to NASH [27-28]. Although the true natural history of the disease is not fully understood. Therefore, the effect of diabetes on early NAFLD patients was excluded in this study, so as to make the study more rigorous.

In this study,we found that there was no statistical difference in the gender between our two groups. However, there are different opinions on whether gender is related to the incidence of NAFLD. Many scholars have found that the prevalence of NAFLD is higher in men than in women, which may be due to the role of female estrogen in inhibiting the production of visceral fat [29-30] . In addition, there are also reports that there is no significant difference between male and female morbidity in India and Malaysia [31].At present, it is not clear which age stage has the greatest impact on NAFLD. Some scholars have found that young people are the most likely to develop NAFLD[32].Some scholars also believe that the incidence of NAFLD increases significantly with age, especially in women after the age of 50 [33].This study included 27 patients from

adolescents to the elderly , and found that there was no statistical difference between the age and the prevalence of NAFLD,possibly due to the small sample size of this study.Numerous studies have found that obesity is an independent risk factor for patients with NAFLD[31-32,34]and the incidence of NAFLD is proportional to BMI [35-36]. Our study are consistent with the results reported so far, confirming once again that high BMI is closely related to the incidence of NAFLD.

Liver function indexs is currently the most widely used serum marker for clinical evaluation and diagnosis of liver injury.The higher the ALT and AST levels, the more severe the liver tissue damage [37-38].However, all patients included in this study were early NAFLD patients with normal liver function who were unwilling to receive liver biopsy, so there was no statistically significant difference in liver function between the two groups. The purpose of this study was also to find more sensitive serum metabolites that could predict the development trend of NAFLD disease.

Our study found no apparent relationship between smoking and NAFLD, which is similar to some literature reports [39-40]. However, Liu[41] found that active smoking is associated with NAFLD progression, passive smoking has also been found to increase the risk of NAFLD in patients compared to active smoking [42].Therefore, more researchs are needed to determine the exact relationship between smoking and NAFLD. It was reported that appropriate drinking can reduce prevalence of NAFLD, improve lipid metabolism and delay the progress of diabetes [43-44], however, some literatures have found that moderate alcohol consumption can aggravate the progression of liver fibrosis [45-46], from which we found that there is no definite relationship between drinking and NAFLD. In addition, the influence of alcohol consumption dose, type, drinking time and drinking style on fatty liver disease was still unclear[46-47],which needed to be confirmed by a larger sample size.

This study found that high TG, high TC, high LDL-C and low HDL-C were closely related to NAFLD disease, which was consistent with Malik [48]. However, Abdul[31]found that there is no obvious relationship between NAFLD and LDL-C,TC, but a significant relationship with TG. In addition, Fang[49]used the TG / HDL-C ratio as a predictor of NAFLD. From this, it can be found that whether lipid level can be used as a predictor of the progression of NAFLD is still controversial. Furthermore, it is debatable whether different ethnic groups, different age groups, and different dietary habits influence the results.

It has been reported that liver lipotoxicity of free fatty acids, cholesterol, ceramide and lysophosphatidylcholine is the main reason for the progress of NAFLD. The simple accumulation of triglycerides may not lead to NAFLD[50-52].The type of accumulated lipids may determine the severity and development trend of NAFLD. Besides, we found that LysoPC, LysoPE, Phenylalanine, Oleic acid and Tryptophan in NAFLD combined with hepatitis patients were abnormal[53-56].It confirmed that these serum indicators promoted the development of NAFLD in whole process and the early detection of which owned huge clinical significance. In addition, some new metabolites of high diagnostic accuracy (AUC > 0.9) related to early NAFLD patients were found. They were Leukotriene C5, 1-Alkyl-2-acylglycerophosphoethanolamine, L-Lysine, Indole, and Homovanillic acid.

The contents of metabolites in phenylalanine metabolism pathway all raised (Fig.2). Phenylalanine and its related metabolites were mainly metabolized in liver[57-59].Some research showed the raise of phenylalanine content were highly correlated with obesity and liver steatosis[60-62]. Other studies have found that phenylalanine levels rise significantly in people with T2DM, especially after after a standardized meal[63].What's more, Palmer et al[64]found that logistic regression analysis from 72 high and 75 low SI subjects revealed significantly decreased glycine and increased valine, leucine, phenylalanine, and combined glutamine and glutamate in insulin-resistant subjects.So, deterioration of liver function in NAFLD patients may cause to the decrease of phenylalanine metabolism. Finally, the phenylalanine and its related metabolites accumulated in liver and serum. However, there are still some limitations in our research: 1. In this article We used serum from 224 patients for experiment. So, the research subjects should be enlarged for more accurate and significantly different data. What's more, patient grouping needs to be more detailed. Different age groups, and different dietary habits should be concerned. 2. We used ultrasound and not histology or MRI for diagnosing NAFLD, so there may be potential human judgment errors. We should strengthen our communication skills with patients and try to persuade them to take liver biopsy or MRI. 3. Liver function and blood lipids are the most commonly used observations and predictions of changes in patients' condition, and non-invasive serological indicators for assessing patients' symptoms have a very broad basis for use. However, many studies have found and confirmed that liver function and blood lipid can only be used as an end-point observation index, rather than a process judgment index, which has certain limitations on the diagnosis, judgment and prediction of the disease progression of fatty liver, especially early fatty liver. Finding noninvasive serological indicators that can accurately diagnose early fatty liver disease and sensitively judge the progress of fatty liver disease is still a problem in front of us.was much higher than the indicators used in clinical which may be helpful for the diagnosis of early NAFLD patients.

Conclusions

15 metabolites with high diagnosis value were found from our metabolomics study in early NAFLD patients. These new biomarkers were of great clinical significance.

Abbreviations

NAFLD — Nonalcoholic Fatty Liver Disease

TBA — total bile acid

CHE — cholinesterase

TB — total bilirubin

DB — direct bilirubin

TP — total protein

ALB — albumin

γ-GT — γ-glutamyltransferase

ALP — alkaline phosphatase

AST — Aspartate aminotransferase

ALT — alanine aminotransferase

HDL — high density lipoprotein

LDL — low density lipoprotein

TC — cholesterol

TG — triglyceride

GLU — glucose in urine

PCA — principal component analysis

OPLS-DA — orthogonal partial least squares discriminant analysis

VIP — variable importance in projection

LC-MS — Liquid chromatography mass spectrometry

Declarations

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Authors' contributions:

Study concept and design: YJ and YJ. Acquisition of data: CH, TW, LC and LZ. Analysis and interpretation of data: CH and JZ. Drafting of the manuscript: CH and JZ. Critical revision of the manuscript for important intellectual content: all the authors. Statistical analysis: CH.

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Availability of data and material:

All data are included in this article.

Ethics approval and consent to participate:

The experimental protocol was approved by the Human Ethics Committee of Putuo Hospital. Written informed consent was obtained from individual or guardian participants.

Consent for publication:

Consent for publication has been obtained from all authors.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Table.1 Baseline characteristics and serum biochemical indexes of subjects

Characteristics	NAFLD (n=112)	Control (n=112)	P value
Gender Male	68	62	
Female	44	48	
Age	46±14	41±15	0.538
Height(cm)	169.42±8.73	170.59±8.07	0.615
Weight(kg)	77.55±13.49	62.37±8.05	0.001**
BMI(kg/m^2)	27.1(24-28.55)	21.46(20.57-22.54)	0.001**
Diastolic pressure (mmHg)	125.41(120-130)	123(59(115-132)	0.375
Systolic pressure (mmHg)	79.74(75-85)	77.63(74-81)	0.183
Waist circumference (cm)	91.75±11.76	72.04±5.96	0.001
Smoking (n)	62	83	0.154
Drinking (n)	76	69	0.776
Blood glucose (mmol/L)	5.12±0.35	5.21±0.42	0.38
TB(umol/L)	12.74±2.54	12.89±3.62	0.862
DB(umol/L)	2.32±0.5	2.23±0.95	0.669
TP(g/L)	73.52±4.71	73.11±5.4	0.769
ALB(umol/L)	41.85±2.99	41.37±5.46	0.689
TB(umol/L)	3.67±2.27	3.85±2.27	0.765
CHE(U/L)	8400.78±1353.66	7694.52±1640.95	0.09
ALT(U/L)	20.67±11.67	28.04±19.13	0.093
AST(U/L)	23.93±6.49	27.7±13.02	0.183
ALP(U/L)	76.96±18.08	84.33±22.15	0.186
γ-GT(U/L)	23.74±11.8	31.59±22.75	0.118
TCmmol/L)	5.67±1.22	4.57±0.8	0.001**
TG(mmol/L)	0.83±0.96	1.24±0.56	0.008*
HDL-C(mmol/L)	0.98±0.18	1.49±0.35	0.002*

LDL-C(mmol/L)	4.89±0.73	3.65±1.01	0.002*

Table.2 Efficiency comparison of diagnostic indicators

No.	Name	AUC	95% CI		Sensitivity	Specificity
			Lower limit	Upper limit		
1	LysoPC(20:3(8Z,11Z,14Z))	0.97	0.933	1	85.19	100.00
2	Succinic acid	0.963	0.917	1	100.00	88.89
3	LysoPC(22:5(7Z,10Z,13Z,16Z,19Z))	0.949	0.896	1	96.30	85.19
4	Indole	0.947	0.881	1	88.89	96.30
5	LysoPC(22:4(7Z,10Z,13Z,16Z))	0.942	0.887	0.998	81.48	92.59
6	Oleic acid	0.938	0.878	0.998	85.19	92.59
7	Desaminotyrosine	0.926	0.849	1	81.48	92.59
8	L-Phenylalanine	0.918	0.838	0.997	92.59	81.48
9	L-Tryptophan	0.915	0.825	1	85.19	96.30
10	LysoPE(22:2(13Z,16Z)/0:0)	0.915	0.837	0.993	85.19	85.19
11	Leukotriene C5	0.909	0.831	0.988	92.59	81.48
12	1-Alkyl-2-acylglycerophosphoethanolamine	0.905	0.829	0.981	81.48	81.48
13	LysoPE(0:0/20:3(5Z,8Z,11Z))	0.905	0.83	0.981	77.78	88.89
14	L-Lysine	0.905	0.815	0.995	85.19	88.89
15	LysoPE(0:0/22:4(7Z,10Z,13Z,16Z))	0.9	0.82	0.979	96.30	70.37
16	Homovanillic acid	0.9	0.801	0.999	88.89	96.30
17	Sulfuric acid	0.894	0.81	0.979	77.78	88.89
18	LysoPE(16:1(9Z)/0:0)	0.893	0.806	0.98	92.59	74.07
19	Coumarone	0.886	0.797	0.975	77.78	88.89
20	LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))	0.885	0.796	0.974	77.78	88.89
21	LysoPC(20:4(8Z,11Z,14Z,17Z))	0.879	0.784	0.975	81.48	85.19
22	LysoPE(0:0/22:5(4Z,7Z,10Z,13Z,16Z))	0.878	0.78	0.976	74.07	92.59
23	Benzoic acid	0.877	0.784	0.969	77.78	88.89
24	LysoPC(20:2(11Z,14Z))	0.87	0.768	0.972	74.07	100.00
25	LysoPE(0:0/24:6(6Z,9Z,12Z,15Z,18Z,21Z))	0.87	0.761	0.978	85.19	81.48
26	LysoPC(20:4(5Z,8Z,11Z,14Z))	0.868	0.771	0.965	81.48	85.19
27	1-arachidonoyl-sn-glycero-3-phosphoethanolamine	0.867	0.774	0.96	88.89	70.37

28	1-Acyl-sn-glycero-3-phosphoethanolamine	0.863	0.745	0.98	74.07	96.30
29	L-methionine	0.86	0.758	0.963	85.19	77.78
30	4-Hydroxycinnamic acid	0.855	0.753	0.956	70.37	92.59
31	Arachidonic acid	0.85	0.75	0.951	85.19	70.37
32	L-TYROSINE	0.846	0.741	0.952	70.37	92.59
33	L-Lactic Acid	0.845	0.738	0.952	92.59	66.67
34	Palmitic Acid	0.844	0.72	0.967	85.19	88.89
35	LysoPE(20:1(11Z)/0:0)	0.841	0.73	0.952	85.19	77.78
36	LysoPC(18:2(9Z,12Z))	0.833	0.724	0.941	70.37	92.59
37	LysoPC(18:1(9Z))	0.826	0.711	0.94	70.37	96.30
38	Ethyl acetate	0.824	0.709	0.94	74.07	88.89
39	1-[(9Z)-hexadecenoyl]-sn-glycero-3-phosphocholine	0.822	0.708	0.935	96.30	59.26
40	LysoPC(15:0)	0.82	0.694	0.947	77.78	92.59
41	Glycerylphosphorylcholine	0.818	0.688	0.947	77.78	92.59
42	LysoPC(18:3(9Z,12Z,15Z))	0.816	0.706	0.926	74.07	77.78
43	Uric Acid	0.815	0.69	0.939	66.67	92.59
44	2-Acyl-sn-glycero-3-phosphoethanolamine	0.811	0.694	0.928	81.48	70.37
45	Stearic acid	0.793	0.664	0.922	70.37	85.19
46	LysoPE(0:0/20:0)	0.785	0.65	0.92	77.78	74.07
47	L-Palmitoylcarnitine	0.761	0.628	0.895	70.37	85.19
48	LysoPE(0:0/16:0)	0.76	0.626	0.894	77.78	66.67
49	2-linoleoyl-sn-glycero-3-phosphoethanolamine	0.749	0.618	0.88	66.67	77.78
50	Platelet-activating factor	0.708	0.563	0.852	51.85	96.30
51	L-Carnitine	0.705	0.568	0.842	59.26	74.07
52	1-heptadecanoyl-sn-glycero-3-phosphocholine	0.672	0.52	0.824	55.56	85.19
53	L-Valine	0.236	0.102	0.37	14.81	33.33
54	L-Pyroglutamic acid	0.091	0.007	0.174	22.22	7.41
55	Citric acid	0.056	0	0.131	3.70	7.41

Figures

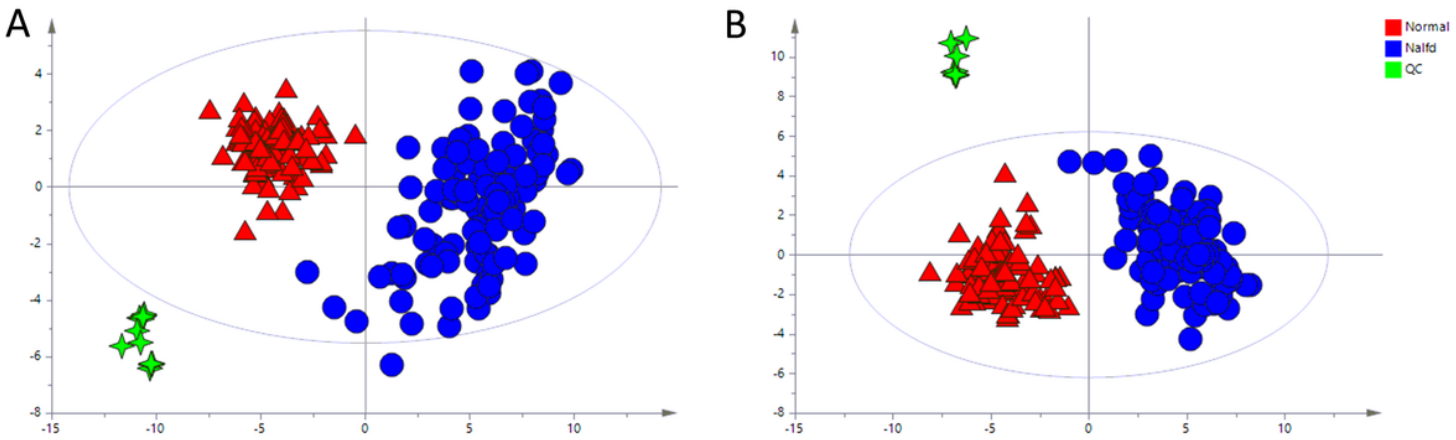

Figure 1

PCA of normal and NAFLDs in positive and negative ion mode. (A) PCA in positive mode; (B) PCA in negative mode (Normal, n=112; NAFLD, n=112)

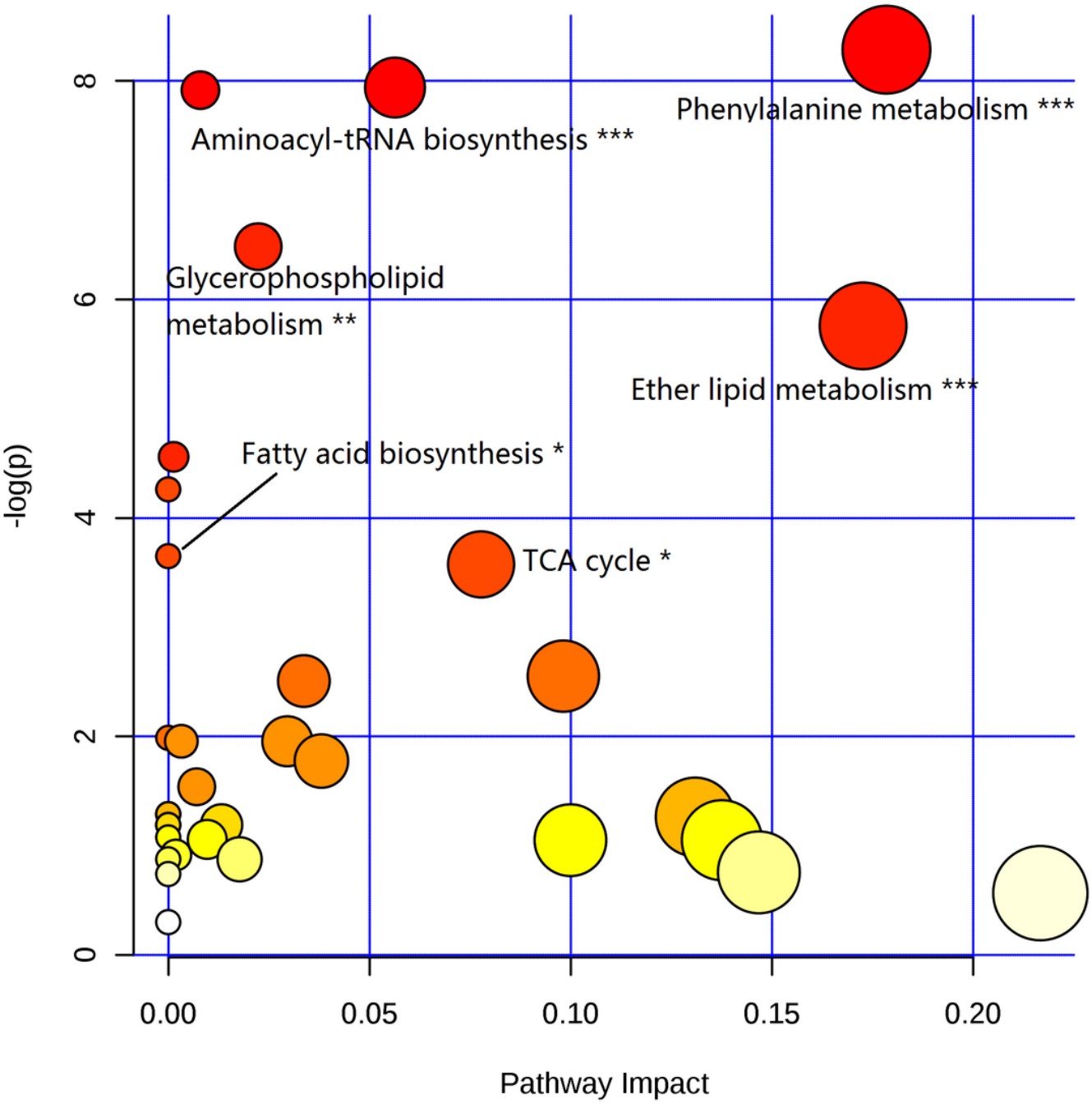

Figure 2

Pathway analysis of significant altered metabolites in study participants.

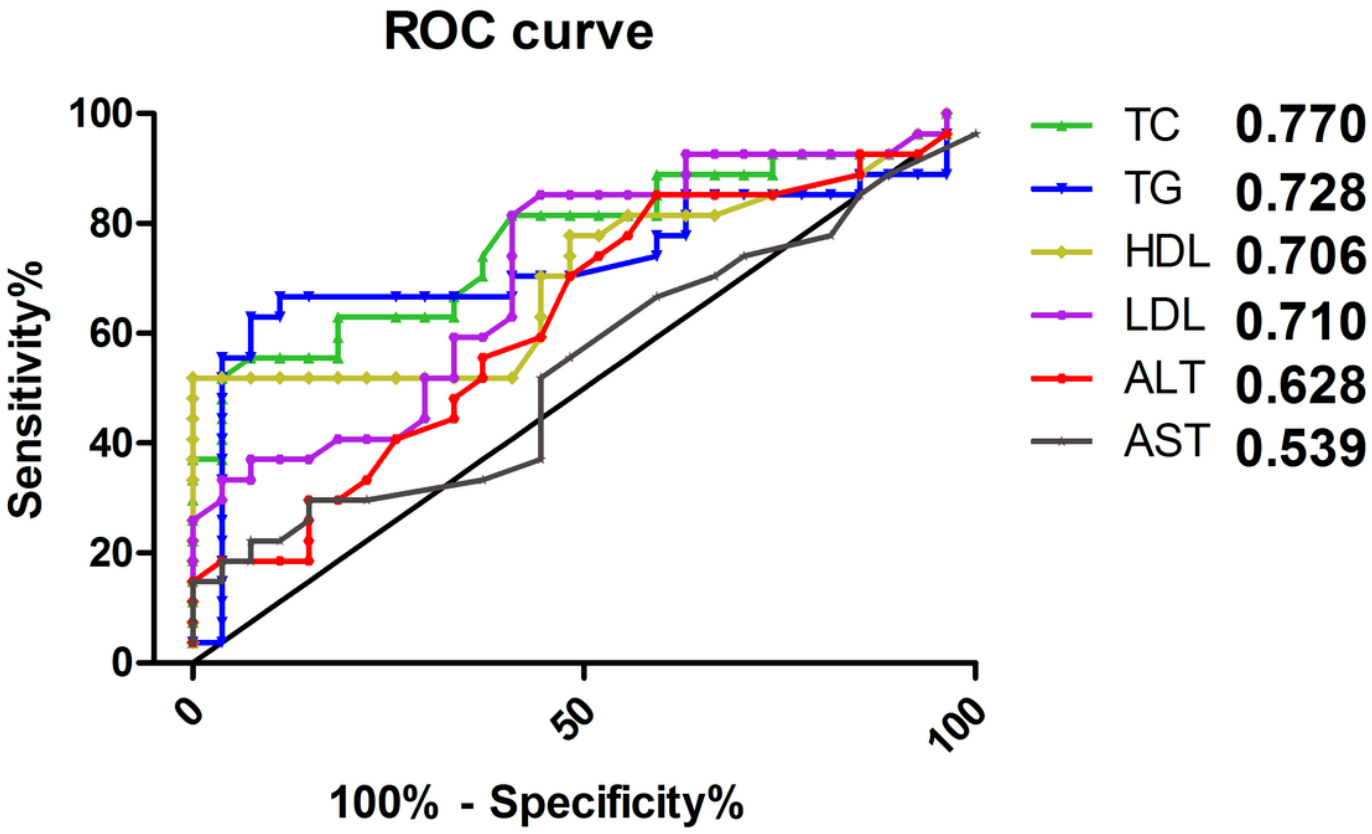

Figure 3

ROC curve of clinical indicators.

Supplementary Files

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