European Review for Medical and Pharmacological Sciences 2020; 24: 7433-7441

A targeted metabolomic profiling of plasma acylcarnitines in nonalcoholic fatty liver disease

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Abstract. – OBJECTIVE: Nonalcoholic fatty liver disease (NAFLD) has become a common liver disorder caused by lipid accumulation and insulin resistance (IR). Acylcarnitines have become a new biomarker of IR. However, their roles in NAFLD are still poorly studied. Thus, we performed a targeted metabolomic analysis to study the level of plasma acylcarnitines in patients with NAFLD.

PATIENTS AND METHODS: The levels of 34 plasma acylcarnitines were measured by a targeted metabolomic approach in NAFLD patients (n = 50) and in healthy control subjects (n = 50) by liquid chromatography–tandem mass spectrometry. Detailed demographic and clinical characteristics of all subjects were also analyzed.

RESULTS: The clinical presentation of IR was identified in the NAFLD group but not in the healthy control group. Significant differences were found in the levels of several short-, medium- and long-chain acylcarnitines. A high degree of correlation (r>0.7) was found between even-numbered-carbon long-chain acylcarnitines in NAFLD patients. The area under the receiver operator characteristic of long-chain acylcarnitines, especially C20 (AUC=0.952), C16:1 (AUC=0.949) and C14:1OH (AUC=0.944) acylcarnitines, was greater in NAFLD patients than in healthy control subjects.

CONCLUSIONS: The accumulation and disorders of acylcarnitines are associated with NAFLD. A positive correlation between even-numbered-carbon long-chain acylcarnitines was found, and these even-numbered-carbon long-chain acylcarnitines. could be used as potential novel screening markers for nonalcoholic fatty liver disease.

Key Words:
Nonalcoholic fatty liver disease, Metabolic syndrome, Metabolomic profiling, Acylcarnitine.

Abbreviations

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; AUC = area under the receiver operator characteristic curve; BMI = body mass index; CAP = controlled attenuation parameter; CoA = acyl-coenzyme A; FBG = fasting blood glucose; FINS = fasting insulin; HbA1C = hemoglobin A1C; HCC = hepatocellular carcinoma; HDL = high-density lipoprotein cholesterol; HOMA-IR = homeostasis model assessment of insulin resistance; LC-MS = liquid chromatography-tandem mass spectrometry; LDL = low-density lipoprotein cholesterol; LSM = liver stiffness measurement; MetS = metabolic syndrome; NAFLD = nonalcoholic fatty liver disease; UA=uric acid; TG = triglyceride; TP = total protein; WHR = waist-to-hip ratio; γ-GT = glutamyl transpeptidase.

Introduction

With the global epidemic of obesity and metabolic syndrome (MetS), nonalcoholic fatty liver disease (NAFLD) has become the leading cause of liver burden, affecting 25% of the general population[1,2]. NAFLD encompasses a spectrum of multiple stages, from simple steatosis, nonalcoholic steatohepatitis (NASH), and fibrosis to cirrhosis. Compared with the general population, patients with NASH and fibrosis, the

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more severe form of NAFLD, are at high risk of end-stage liver disease and hepatocellular carcinoma (HCC), similar to other chronic liver diseases. NAFLD has also emerged as the second leading etiology for liver transplantation in the United States [3]. The increase in chronic liver disease caused by NAFLD is concerning, and it appears that the incidence will increase over the next 20 years[1,4]. Although ultrasound, transient elastography and pathology are used to identify hepatic steatosis, many patients are found to be in the advanced stage. It is generally considered that patients may be asymptomatic in the early stage of NAFLD[5]. Even 20% of NASH patients have aminotransferases in the normal range[6,7]. For this reason, more novel biomarkers must be identified to improve our ability to identify patients at the early stage of NAFLD.

Impaired fatty acid β-oxidation, especially disorders of the oxidation of long-chain fatty acids and lipid overload in mitochondria, is considered to be the main mechanism of lipid accumulation and insulin resistance[8]. The β-oxidation of fatty acids is mainly carried out in the liver and muscle. Fatty acids are first catalyzed by acyl coenzyme A (CoA) synthetase from the endoplasmic reticulum and mitochondrial outer membrane to produce lipid acyl-CoA[9]. Activated lipoyl CoA is transported into mitochondria by means of carnitine. Acylcarnitine is an intermediate product of carnitine metabolism and has become a new index of abnormal fatty acid oxidation[10].

However, it is still poorly understood whether differences in acylcarnitine metabolism occur in NAFLD patients and whether they can be used to detect NAFLD patients in the early stage of the disease[11]. A targeted metabonomic analysis offers a new avenue to measure the absolute content of metabolites[12], and this prompted us to study the level of plasma acylcarnitines in patients with NAFLD. More specifically, we explored the changes in and diagnostic prospects of acylcarnitines in different subjects to provide possible help for finding early-stage patients with NAFLD.

Patients and Methods

Study Design and Patients

A total of 100 adults were admitted to the Metabolic Management Center or Cardiovascular and Cerebrovascular Disease Management Center of Tianjin Fourth Central Hospital, Characteristic Medical Center of People's Armed Police Force, and Beijing Friendship Hospital. All patients were recruited to participate in the study after written informed consent was obtained and reviewed by the ethics committee. This study was approved by the Ethics Review Committee of Tianjin Fourth Central Hospital and Beijing Friendship Hospital and was consistent with the rules of the Declaration of Helsinki. This study was registered with ClinicalTrials.gov (NCT03635541).

Because of the strong association between demographic characteristics and metabolites[13], we conducted pairwise comparisons of two groups. The patients with NAFLD and the healthy controls were matched for sex, and the age difference was controlled within 1 year.

NAFLD patients and insulin resistance were determined according to the clinical practice guidelines for the management of NAFLD from the European Association for the Study of the Liver[14]. The diagnosis of NAFLD was confirmed pathologically with liver biopsy. None of the subjects had a drinking history (male<30 g/d, female <20 g/d)[7]. Exclusion: subjects with hepatitis virus infection, autoimmune liver disease, drug-induced liver injury, or other liver diseases. The members of the healthy control group were recruited from the physical examination center of the same hospital and had no history of any liver disease.

Clinical Records and Plasma Preservation

Various detailed demographic and baseline data were collected from all subjects, and fasting blood samples were taken. A history of MetS and the presence of type 2 diabetes mellitus (T2DM) were defined according to self-reported history or the use of medications.

For all subjects, the following clinical baseline data were recorded: age, sex, height (accurate to 0.1 cm), weight (accurate to 0.1 kg), waist circumference (accurate to 0.1 cm), and hip circumference (accurate to 0.1 cm). The waist-to-hip ratio (WHR) was calculated. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), total protein (TP), total bilirubin (TBIL), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), fasting blood glucose (FBG), fasting insulin (FINS) and hemoglobin A1C (HbA1C) were also collected from medical records.

Body mass index (BMI) was calculated as follows: BMI = weight (kg)/height $(cm)^2$. The Homeostasis Model Assessment of Insulin Re-

sistance (HOMA-IR) was calculated in accordance with the following formula: FBG (mmol/L) ×FINS (μlU/mL)/22.5.

Ultrasonography

A diagnostic ultrasound scanner (Toshiba SSA-580A, Otawara, Japan) was used by professional radiologists to scan for the presence of NAFLD.

Additionally, the FibroScan 502 (Echosens, Paris, France) with an M-probe was used to assess the degree of steatosis for all subjects. The controlled attenuation parameter (CAP) and liver stiffness measurement (LSM) were recorded.

Measurement of Plasma Acylcarnitine

An Agilent 6410B QQQ mass spectrometer equipped with an Agilent 1260 Series HPLC (Santa Clara, CA, USA) was used to simultaneously quantify free carnitine and acylcarnitines. Briefly, serum samples were all collected under standard conditions in the patients' fasting state and stored at -80°C. For further analysis, samples were thawed, vortexed and centrifuged at 4°C for 15 min at 16000 relative centrifugal force (rcf). Ten milliliters of the supernatants were mixed with 90 mL of methanol containing 360-fold diluted internal standards (containing deuterium-labeled carnitine and acylcarnitines). After drying the plasma samples under a nitrogen stream. Filters were dried, and metabolites, as well as internal standards, were extracted with acetonitrile/water (4:1). After centrifugation of the solution through the filter plate (15 min, 16,000 rcf), the extracts were diluted with mass spectrometry running solvent. Twenty microliters of the final extracts were subjected to liquid chromatography–tandem mass spectrometry (LC-MS) in negative electrospray ionization mode and one in positive electrospray ionization mode. Metabolites were quantified by multiple reactions monitoring detection in reference to stable isotope-labeled internal standards. Mass Hunter software (version B.03.01, Santa Clara, CA, USA) was used for data evaluation and the quantification of metabolite concentrations[15].

Statistical Analysis

All variables were analyzed with a Shapiro-Wilk test ($p < 0.05$) for distribution and Levene's test ($p < 0.05$) for equal variances. The levels of identified acylcarnitine were log-transformed to meet the normal distribution. Variables are presented as the mean ± the standard error or the median (1/4 median, 3/4 median). For normally distributed data with equal variance, a paired Student's t-test was used; otherwise, the Mann-Whitney U test was performed to compare the clinical characteristics and acylcarnitine differences between NAFLD subjects and healthy control subjects. A Pearson correlation was used to analyze the correlation between each of the plasma acylcarnitines in the two groups. A receiver operator characteristic (ROC) curve was used to analyze the area under the ROC curve (AUC) of different acylcarnitines in distinguishing NAFLD patients from healthy controls. Data were analyzed using Statistical Product and Service Solutions software (Chicago, IL, USA), version 22.0, and R language, version 3.0 (http://R-project.org/). A two-sided p α-level of 0.05 indicated a significant difference.

Results

Clinical Data of NAFLD Patients and Healthy Controls

To dissociate the confounding effects of age and sex on the clinical characteristics and acylcarnitine profile, we recruited subjects with NAFLD and healthy controls who had no history of liver disease, and the two groups of subjects were matched for age and sex (Table I). Baseline characteristics were compared between the two groups. More subjects in the NAFLD group than in the healthy control group had a history of MetS and T2DM. As expected, we also observed that the subjects in the NAFLD group showed higher levels of BMI, ALT, AST, γ-GT, UA, FBG, FINS, HOMA-IR, LDL, TG, TC and CAP (all $p < 0.05$). No significant difference was observed for the other comparisons ($p > 0.05$), as shown in Table I.

Metabolomics Signature of Acylcarnitines

Plasma acylcarnitine levels below the lower limit of detection were excluded. Ultimately, thirty-four plasma acylcarnitines were included in this study. As shown in Figure 1, acylcarnitines were compared in NAFLD patients and healthy controls. Interestingly, the identified long-chain acylcarnitines (carbon chains ≥16) all had even-numbered-carbon chains. The levels of long-chain acylcarnitines in NAFLD patients were higher than those in the healthy control group. Significant differences were found in several short- and medium-chain acylcarnitines (carbon chains ≤6 and 7–14, respectively) between the two groups. Elevated levels of short-chain acyl-

Table I. Baseline characteristics of NAFLD patients and healthy control subjects.

Characteristic	NAFLD (n = 50)	Healthy control (n = 50)	*p*-value
Age (yr)	44 ± 14	44 ± 14	1.000
Male (n)	27	27	1.000
History of MetS (n, %)	27 (54%)	9 (18%)	< 0.001
History of T2DM (n, %)	19 (38%)	4 (8%)	< 0.001
WHR	0.94 ± 0.04	1.07 ± 0.06	< 0.001
BMI (kg/m^2)	26.81 ± 3.43	23.73 ± 1.95	< 0.001
ALT (U/L)	88.9 ± 10.76	27.36 ± 9.76	< 0.001
AST (U/L)	37.17 ± 12.74	21.41 ± 13.75	< 0.001
γ-GT (U/L)	40.25 ± 23.66	27.39 ± 13.04	0.001
ALP (U/L)	73.43 ± 17.05	79.1 ± 16.02	0.090
TBil (μmol/L)	12.63 ± 4.04	10.99 ± 4.92	0.072
DBil (μmol/L)	3.62 ± 1.72	3.05 ± 1.34	0.068
UA (μmol/L)	321.28 ± 96.25	249.63 ± 99.46	< 0.001
FBG (μmol/L)	7.44 ± 2.05	5.25 ± 0.83	< 0.001
FINS (μlU/mL)	14.7 (13.1-19.4)	12.9 (9.6-13.3)	< 0.001
HOMA-IR (mmol*μlU/mL)	5.24 ± 2.09	2.06 ± 0.53	< 0.001
HbA1C (%)	6.12 ± 0.62	4.83 ± 0.47	< 0.001
HDL (μmol/L)	1.28 ± 0.31	1.33 ± 0.37	0.466
LDL (μmol/L)	3.53 ± 0.77	1.94 ± 0.95	< 0.001
TG (μmol/L)	2.49 ± 1.64	1.32 ± 0.41	< 0.001
TC (μmol/L)	5.32 ± 0.77	4.32 ± 0.83	< 0.001
LSM (kPa)	7.47 ± 2.33	5.41 ± 2.49	< 0.001
CAP (dB/m)	315.72 ± 38.87	217.46 ± 21.41	< 0.001

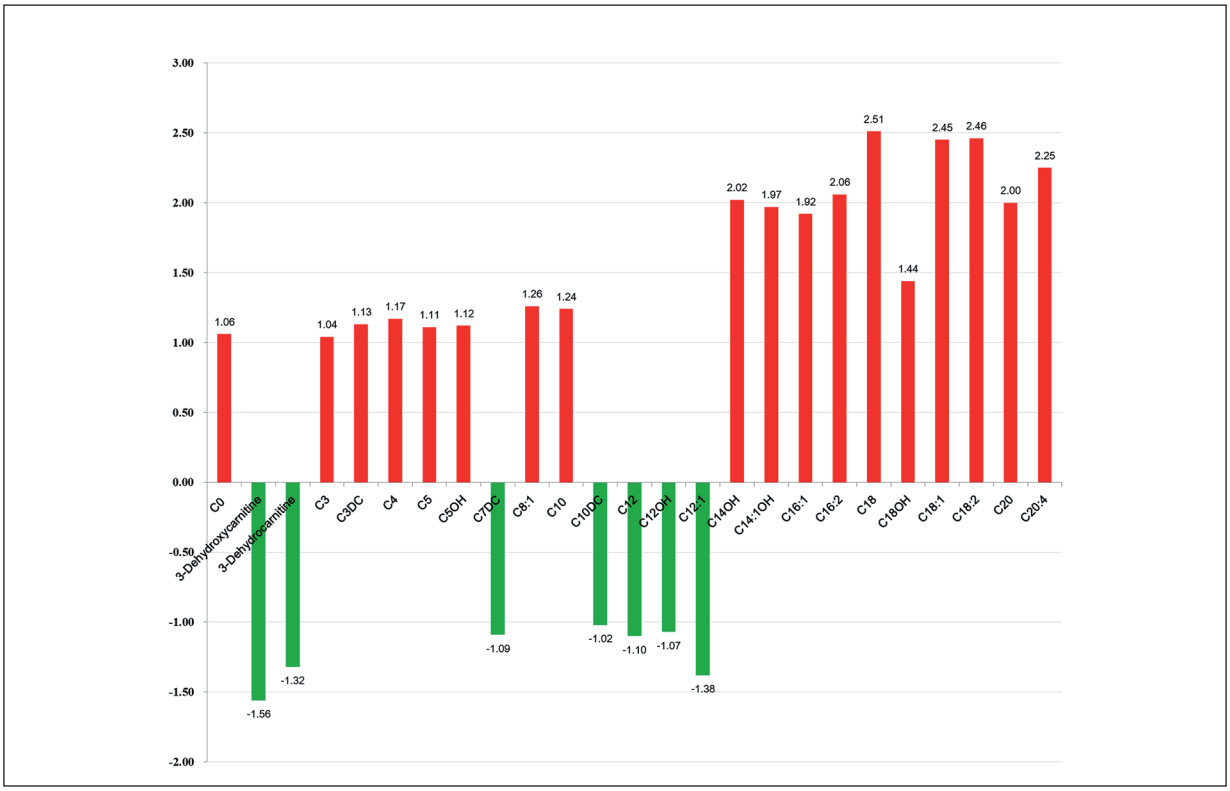

Figure 1. Comparison of the level of plasma acylcarnitines between NAFLD subjects and healthy control subjects. Red represents increased levels of plasma acylcarnitines in the NAFLD group. Green represents decreased levels of plasma acylcarnitines in the NAFLD group.

carnitine (carbon chains ≤6) were also found in NAFLD patients, but not 3-dehydroxy carnitine or 3-dehydro carnitine. Among medium-chain acylcarnitines, the levels of C8:1, C10, C14OH, and C14:1OH acylcarnitines were elevated, while the levels of C7DC, C10DC, and acylcarnitines with 12 carbon atoms were significantly reduced. Table II provides a more detailed comparison of the acylcarnitine profile.

Correlations Between Acylcarnitines

The correlations between the included acylcarnitines were explored in the two groups, as shown in Figure 2. A high degree of correlation (r>0.7) was found between long-chain acylcarnitines in NAFLD patients (Figure 2A). In contrast, this trend did not appear in the healthy control group. Interestingly, no significant correlations were observed between most acylcarnitines in the healthy control group (Figure 2B). These results suggested that the accumulation and disorders of long-chain acylcarnitines are related to NAFLD.

Potential Diagnostic Value of Acylcarnitines

To analyze the potential diagnostic value of acylcarnitines in plasma for NAFLD, an ROC analysis was established. In the ROC curve analysis, acylcarnitines with an area under the ROC (AUC) greater than 0.8 were screened. The AUC was greater for long-chain acylcarnitines, especially C20 (AUC=0.952), C16:1 (AUC=0.949) and C14:1OH (AUC=0.944) acylcarnitines, than for short- and medium-chain acylcarnitines. Acylcarnitines with AUCs ≥ 0.9 are shown in Figure 3.

Table II. Baseline characteristics of NAFLD patients and healthy control subjects.

No.	Acylcarnitine	NAFLD (n = 50)	Healthy controls (n = 50)	p-value
1	C0	61.8 (49.1,74.6)	58.2 (42.8,71.3)	< 0.001
2	3-Dehydroxycarnitine	0.27 (0.25,0.28)	0.42 (0.39,0.44)	< 0.001
3	3-Dehydrocarnitine	2.08 (1.99-2.22)	2.75 (2.67-2.82)	< 0.001
4	C2	11.3 (10.9,11.6)	10.9 (10.7,11.2)	0.065
5	C3	0.48 (0.46,0.51)	0.46 (0.45,0.47)	0.022
6	C3DC (10^{-2})	4.82 (4.66,4.97)	4.25 (4.17,4.36)	< 0.001
7	C4	0.21 (0.19,0.24)	0.18 (0.16,0.23)	< 0.001
8	C5 (10^{-2})	6.59 (6.35,6.83)	5.95 (5.83,6.07)	< 0.001
9	C5OH (10^{-2})	0.57 (0.55,0.58)	0.51 (0.50,0.52)	< 0.001
10	C5:1 (10^{-2})	0.94 (0.91,0.97)	0.97 (0.95,1.00)	0.121
11	C6 (10^{-2})	3.59 (3.49,3.70)	3.65 (3.58,3.73)	0.680
12	C6OH (10^{-2})	1.22 (1.17,1.26)	1.27 (1.24,1.29)	0.110
13	C6DC (10^{-2})	0.53 (0.51,0.57)	0.54 (0.52,0.56)	0.171
14	C7DC (10^{-2})	0.22 (0.21,0.23)	0.24 (0.24,0.25)	< 0.001
15	C8	0.22 (0.21,0.23)	0.19 (0.18,0.20)	0.140
16	C8:1	0.54 (0.51,0.57)	0.43 (0.42,0.44)	< 0.001
17	C10	0.31 (0.29,0.32)	0.25 (0.24,0.26)	< 0.001
18	C10DC (10^{-2})	0.47 (0.46,0.49)	0.48 (0.47,0.49)	0.036
19	C12 (10^{-2})	7.45 (7.14,7.76)	8.17 (7.96,8.39)	< 0.001
20	C12OH (10^{-2})	0.67 (0.65,0.70)	0.72 (0.70,0.74)	< 0.001
21	C12:1 (10^{-2})	8.90 (8.47,9.34)	12.34 (11.82,12.65)	< 0.001
22	C12DC (10^{-2})	0.51 (0.48,0.55)	0.60 (0.57,0.63)	0.015
23	C14 (10^{-2})	2.17 (2.08-2.25)	2.19 (2.14-2.24)	0.410
24	C14OH (10^{-2})	1.68 (1.55,1.82)	0.83 (0.80,0.86)	< 0.001
25	C14:1OH (10^{-2})	2.07 (1.91-2.24)	1.05 (1.01,1.09)	< 0.001
26	C16 (10^{-2})	8.80 (8.56,9.05)	8.45 (8.31,8.09)	0.002
27	C16:1 (10^{-2})	6.65 (6.19,7.14)	3.46 (3.32,3.61)	< 0.001
28	C16:2 (10^{-2})	2.94 (2.71,3.18)	1.43 (1.38,1.09)	< 0.001
29	C18 (10^{-2})	11.4 (10.61,12.36)	4.54 (4.36,4.73)	< 0.001
30	C18OH (10^{-2})	0.23 (0.21,0.25)	0.16 (0.14,0.18)	< 0.001
31	C18:1 (10^{-2})	9.91 (8.53,11.53)	4.04 (3.81,4.29)	< 0.001
32	C18:2	0.32 (0.30,0.34)	0.13 (0.12,0.13)	< 0.001
33	C20 (10^{-2})	0.74 (0.69,0.80)	0.37 (0.35,0.38)	< 0.001
34	C20:4 (10^{-2})	0.81 (0.75,0.88)	0.36 (0.34,0.37)	< 0.001

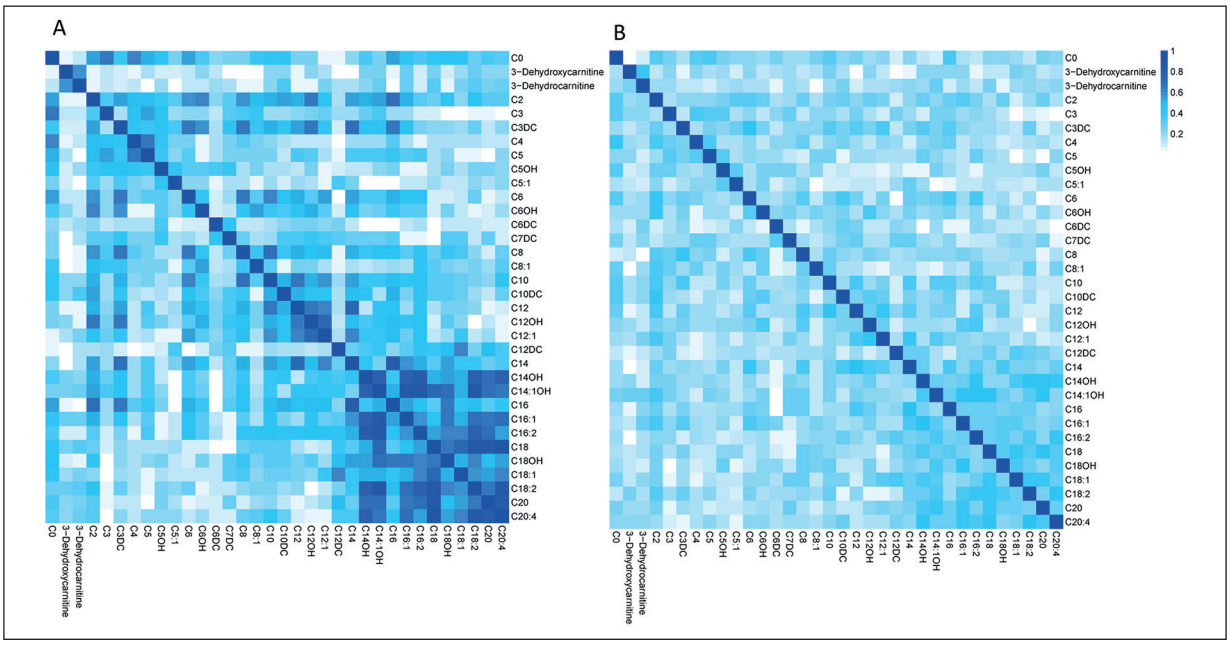

Figure 2. Pearson correlations between acylcarnitines in NAFLD and healthy control subjects. **A,** Correlations between acylcarnitines in the NAFLD patients. **B,** Correlations between acylcarnitines in the healthy control subjects. Dark blue represents the highest correlations, and white represents the lowest relative abundance. The deeper the color, the higher the correlations.

Discussion

To the best of our knowledge, this is the first study to evaluate the changes in the plasma acylcarnitine profile in patients with NAFLD and healthy controls by LC-MC/MS. We found that the accumulation of long-chain acylcarnitines was associated with the occurrence of NAFLD disease. This may provide a new therapeutic target for the prevention and control of NAFLD in the future.

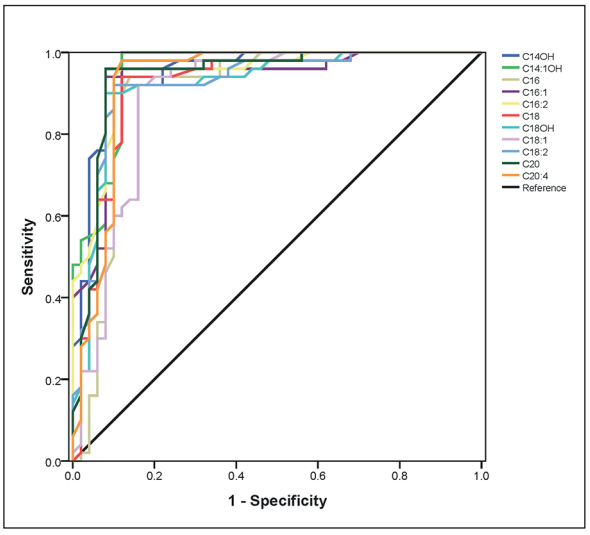

Figure 3. The receiver operator characteristic (ROC) curve using acylcarnitines for the diagnosis of NAFLD. Predictors with an AUC >0.9 are shown in the picture.

We found that the levels of blood fat (TG, LDL, TC) and HOMA-IR in the NAFLD group were significantly higher than those in the healthy control group, and the proportion of patients with T2DM and MetS was also higher in the NAFLD group. All these findings suggest that the NAFLD group had higher levels of IR, which is in congruence with earlier reports[16-18]. Insulin resistance is not only an important factor leading to NAFLD but also the cause of disorders of the oxidation of long-chain fatty acids[19,20]. With the increase in the incidence of NAFLD, research on the mechanism of insulin resistance is deepening. Impaired β-oxidation of fatty acids, especially disorders of the oxidation of long-chain fatty acids and mitochondrial lipid overload, is considered to be the main mechanism of insulin resistance[21]. The β-oxidation of fatty acids is mainly carried out in the mitochondria of the liver and muscle[22]. Acylcarnitine levels have been shown to reflect an early imbalance of fatty acid oxidation and mitochondrial stress in animal studies. Mitochondrial stress caused by mitochondrial lipid overload and incomplete β-oxidation of fatty acids is one of the important causes of insulin resistance and NAFLD.

Significant differences were found in many acylcarnitines between NAFLD subjects and healthy control subjects, especially in medium- and long-chain acylcarnitines. Interestingly, most of them were even-numbered-carbon acylcarnitines. This suggests that the levels of even-numbered-carbon acylcarnitines were correlated with insulin resistance or NAFLD, especially the metabolic disorder of long-chain fatty acids. Previous studies have also found that there is a close association between even-numbered-carbon acylcarnitine disorder and T2DM and lipid accumulation[23,24]. In a lipidomic analysis of plasma and liver biopsy samples, Peng found that increased levels of acylcarnitines can be associated with mitochondrial dysfunction and the progression of NAFLD[25]. Furthermore, we found that there was a positive correlation between each long-chain acylcarnitine in patients with NAFLD. Hardly any of these close relationships were observed in the healthy control group in this study.

Compellingly, we showed that several even-numbered-carbon long-chain acylcarnitines can be a candidate diagnostic marker for NAFLD. The elevated levels of C20, C16:1, and C14:1OH acylcarnitines in plasma were predictive of NAFLD. Of course, the differences between short-chain acylcarnitines in this study cannot be ignored. Unlike even-numbered carbon long-chain acylcarnitines, analyses of odd-numbered-carbon short-chain acylcarnitines are derived from LC-MC/MS. Peng reported that mitochondrial dysfunction contributes to fat accumulation by hepatic cardiolipin and ubiquinone accumulation, increased levels of acylcarnitine, and impaired branched-chain amino acid catabolism[25]. An intervention study has shown that treatment with L-carnitine and nicotinamide riboside can prevent the development of NAFLD by regulating the metabolism of acylcarnitines in mice[26]. Additionally, in a high-fat-diet-fed ApoE-/- and C57BL/6J mouse NAFLD model study with acylcarnitine metabolism disorder, theacrine was found to promote acylcarnitine metabolism and inhibit steatohepatitis by increasing the activity of long-chain acyl coenzyme A dehydrogenase[27]. Whether the mobilization of long-chain fatty acids can be increased by regulating acylcarnitines in patients with NAFLD must be confirmed.

The limitations of the present study should be considered. (1) We included 50 subjects in each group. However, due to the limitations of ethics and patient consent, the healthy control subjects did not undergo liver biopsy. Although they were scanned by FibroScan and ultrasound, the hepatic steatosis in the healthy control group could not be completely ruled out. (2) The subjects involved in the study are adults from East Asia and, therefore, are not representative of the global population, especially Caucasians and Africans. (3) It cannot be determined whether the change in acylcarnitine is the cause or result of NAFLD because of the study design. However, our preliminary data provide a good basis and will stimulate further studies of NAFLD.

Conclusions

Altogether, these data showed that in the current research with new data concerning, for the first time, the pattern of acylcarnitines in NAFLD, we found that the accumulation and disorders of acylcarnitines were associated with NAFLD. In particular, there was a positive correlation between even-numbered-carbon long-chain acylcarnitines in NAFLD patients. These even-numbered-carbon long-chain acylcarnitines could be used as potential novel screening markers for nonalcoholic fatty liver disease. Further investigations are needed to find a treatment that regulates the levels of acylcarnitines in NAFLD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethical Approval and Consent to Participate

This study was approved by Tianjin Fourth Central Hospital

Data Availability Statement

All data generated or analyzed during this study available through the correspondence author Hai Li via email.

Funding

This work was supported by the Tianjin Major Science and Technology Projects (17ZXMFSY00200), High-Priority Health Projects of Tianjin (16KG146); China youth clinical research fund-VG fund (2017-CCA-VG-021), and Tianjin Science and Technology Project (15ZXLCSY00040).

Authors' Contribution

Yue Chang, Fengshi Tian, and Hai Li provided the ideas and designed this study. Yue Chang and Huanming Li drafted the manuscript. Jing He, Kai Chen, and Xiaohui Lin delivered experimental data. Yue Chang and Xiaohui Lin carried out this research and analyzed all the data. All authors have read and contributed to editing this manuscript and agree with the content and presentation of the paper.

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