Metabolomic Analysis Uncovers Energy Supply Disturbance as an Underlying Mechanism of the Development of Alcohol-Associated Liver Cirrhosis

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Alcohol-associated liver disease (ALD) is caused by alcohol metabolism's effects on the liver. The underlying mechanisms from a metabolic view in the development of alcohol-associated liver cirrhosis (ALC) are still elusive. We performed an untargeted serum metabolomic analysis in 14 controls, 16 patients with ALD without cirrhosis (NC), 27 patients with compensated cirrhosis, and 79 patients with decompensated ALC. We identified two metabolic fingerprints associated with ALC development (38 metabolites) and those associated with hepatic decompensation (64 metabolites) in ALC. The cirrhosis-associated fingerprint (eigenmetabolite) showed a better capability to differentiate ALC from NC than the aspartate aminotransferase–to-platelet ratio index score. The eigenmetabolite associated with hepatic decompensation showed an increasing trend during the disease progression and was positively correlated with the Model for End-Stage Liver Disease score. These metabolic fingerprints belong to the metabolites in lipid metabolism, amino acid pathway, and intermediary metabolites in the tricarboxylic acid cycle. *Conclusion:* The metabolomic fingerprints suggest the disturbance of the metabolites associated with cellular energy supply as an underlying mechanism in the development and progression of alcoholic cirrhosis. (*Hepatology Communications* 2021;5:961-975).

The prevalence of alcohol-associated liver disease (ALD) is on the rise, and ALD has become one of the common noninfectious liver diseases worldwide.[1-3] ALD represents a spectrum of histopathological changes ranging from alcoholic steatosis, steatohepatitis, advanced fibrosis, and cirrhosis. Alcoholic steatosis, a reversible process following abstinence, occurs in most if not all heavy drinkers. However, only 20% of these patients develop alcoholic hepatitis and cirrhosis.[4]

Alcohol-associated liver cirrhosis (ALC) is the leading cause of mortality in patients with ALD.[4,5] It accounts for 1% of all deaths worldwide and 50% of cirrhosis-related deaths.[6] The prognosis is poor with 5-year mortality around 85%, especially in those with complications from portal hypertension, such as variceal bleeding, hepatic encephalopathy, and ascites.[7]

Once ingested, alcohol is primarily metabolized by the liver with by-products such as acetaldehyde and reactive oxygen species (ROS), leading to liver

Abbreviations: ALB, albumin; ALC, alcohol-associated liver cirrhosis; ALD, alcohol-associated liver disease; APRI, AST-to-platelet ratio index; AST, aspartate transaminase; ATP, adenosine triphosphate; AUC, area under the curve; BCCA, branched-chain amino acids; CC, compensated cirrhosis; CHE, cholinesterase; CoQ, coenzyme Q; DC, decompensated cirrhosis; INR, international normalized ratio; MAT, methionine adenosyltransferase; MELD, Model for End-Stage Liver Disease; NADH, reduced nicotinamide adenine dinucleotide; NC, noncirrhosis; PCA, principal component analysis; QC, quality control; ROS, reactive oxygen species; SAMe, S-adenosylmethionine; TBil, total bilirubin; TCA, tricarboxylic acid; α-KG, α-ketoglutarate.

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injury.[8,9] Patients with ALD are prominently accompanied by malnutrition, which is caused by the substitution of energy supply from food to alcohol (~7.1 kcal per gram of alcohol) and disturbance of nutrient utilization.[10] Alcohol metabolism also causes alterations in the redox state, high reduced nicotinamide adenine dinucleotide (NADH)/NAD^+ ratio, which interrupts the tricarboxylic acid (TCA) cycle, and suppresses lipid oxidation.[11] The suppression of the TCA cycle results in not only energy supply disruption but also the alteration in intermediary metabolites. These events may increase the vulnerability of hepatocyte injury from acetaldehyde and ROS, due to the need of these metabolites for tissue repair and regeneration.[12] The metabolites that are associated with the progression of ALD and the development of ALC have not been studied extensively. The objectives of this study were to characterize the sera metabolite fingerprints among heavy drinkers who developed ALC, specifically in those with the decompensated state.

Materials and Methods

STUDY DESIGN AND STUDY COHORT

Healthy controls without history of excessive alcohol use and patients with ALD who were seen at the Fifth Medical Center of Chinese PLA General Hospital between January 1, 2015, and December 31, 2017, were enrolled in the study. Patients with other etiologies of chronic liver diseases such as viral hepatitis, cholestatic liver diseases, and autoimmune liver disease were excluded. Baseline demographic and clinical data were obtained. Serum samples were stored at −80°C until the analysis. The diagnostic criteria for ALD as well as its clinical classification are given in Table 1. A total of 136 patients constituted the study cohort: 14 healthy controls (CON), 16 patients with ALD without cirrhosis (NC), 27 cases with compensated ALC, and 79 cases with decompensated ALC.

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TABLE 1. DIAGNOSTIC CRITERIA AND DEFINITIONS FOR ALD

Definitions and Diagnostic Criteria	Descriptions
ALD	Consists of three parts: (1) patients with a history of excessive alcohol consumption of >20 g/day in females and >40 g/day in males over 5 years; (2) patients with liver injury by clinical manifestation, abnormal liver biochemistries, radiographic imaging, and/or histological findings; and (3) other causes of liver diseases (excluded)
Cirrhosis	A condition in which the liver is scarred and permanently damaged based on relevant imaging tests or liver biopsy
Compensated ALD cirrhosis	Consist of two parts: (1) patients diagnosed with ALD; and (2) patients with cirrhosis according to the radiographic imaging or histological findings, without any complications of advanced liver disease
Decompensated ALD cirrhosis	Consist of three parts: (1) patients diagnosed with ALD; (2) patients with cirrhosis according to the radiographic imaging or histological findings; and (3) patients with complications of advanced liver disease including ascites, variceal bleeding, acute kidney injury, hepatorenal syndrome, bacterial infections, and hepatic encephalopathy
Non-cirrhosis	Patients diagnosed with ALD but without the condition of cirrhosis in terms of radiographic imaging or histological findings

Written, informed consent was obtained from all participants. The study was approved by the ethics committees of the Fifth Medical Center of the General Hospital, Beijing.

PREPARATION OF SERUM SAMPLES

Stored serum samples were reconstituted at 4°C, and an aliquot of 200 μL was pipetted into an Eppendorf tube and diluted with a 3-fold quantity of precooled methanol. Samples were deproteinized at 14,000 revolutions per minute (rpm) for 10 minutes in a precooled centrifuge at 4°C. Liquid supernatant of 150 μL was pipetted into an Eppendorf tube and dried in a vacuum centrifuge concentrator. Furthermore, 75 μL of 75% methanol was used to reconstitute. Reconstituted sample was centrifugated at 12,000 rpm for 10 minutes in a precooled centrifuge at 4°C. Liquid supernatant of 50 μL was pipetted into the liner tube in vial for analysis. Quality control (QC) samples were prepared by mixing each sample of 10 μL to be analyzed.

ULTRAHIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–QUADRUPLE TIME-OF-FLIGHT MASS SPECTROMETRY MEASUREMENT

Serum samples were subjected to untargeted metabolomic analysis using ultrahigh-performance liquid chromatography coupled with a high-resolution mass spectrometer according to previously established methods.[13] For the chromatographic column, we used the ZORBOX RRHD C18 analytical column with 2.1-mm inner diameter, 100-mm length, and 1.8-μm particle size (Agilent Technologies, Santa Clara, CA). Column temperature was 30°C and sample temperature was 4°C. For mobile phase A, we used water with 0.1% formic acid in positive mode of quadruple time of flight, and pure water in negative mode; for mobile phase B, we used acetonitrile. The flow rate was set to 0.30 mL/min; sample injection size was 4 μL; and the chromatographic gradient elution conditions were as follows: 0-1 minute, 100% (A); 1-9 minutes, 100%-60% (A); 9-19 minutes, 60%-10% (A); 19-21 minutes, 10%-0% (A); and 21-25 minutes, 100% (B). The nontargeted primary mass spectrometric detection conditions were as follows: mass range of 50-1,200 m/z; gas temperature of 225°C in both positive and negative ionization modes; airflow of 13 L/min; nebulizer of 20 pisg (negative) and 20 pisg (positive); sheath gas temperature of 275°C and sheath gas flow of 12 L/min; electrospray capillary voltage of 3.5 kV in negative ionization mode and 4 kV in positive ionization mode; and nozzle voltage of 2,000 V in the positive and negative ion mode. For the mass correction, we used a reference mass of 121.0509 (Purine, $[C_5H_4N_4H]^+$) and 922.0098 (HP-0921, $[C_{18}H_{18}O_6N_3P_3F_{24}H]^+$) in positive ion mode and 112.9856 (TFANH4, $[C_2O_2F_3]^-$) and 1033.9881 (TFANH4+HP-0921, $[C_{20}H_{22}O_8N_4P_3F_{27}\text{-}NH_4]^-$) in negative ion mode. The QC samples were evenly inserted in each set of the analysis running sequences, to monitor the stability of the large-scale analysis.

DATA PROCESSING AND STATISTICAL ANALYSIS

The peak alignment and data filtering were processed using Mass Profinder (version B.06.00; Agilent Technologies). For the molecular feature extraction, up to 13,000 compounds with peak height above 300

counts were extracted. Metabolomic data were normalized by the inclusion of multiple internal standards and pool calibration–response correction in MetaboAnalyst version 4.0 after being processed in Masshunter Profinder. The normalized data were analyzed using the Wilcoxon Mann-Whitney U test with $P < 0.05$ set as the level of statistical significance. These variables were identified in the Human Metabolome Databases. Descriptive statistics for continuous variables were presented as mean with SD for normally distributed parameters or median with corresponding upper and lower quartiles for nonnormally distributed parameters. For categorical data, numbers and percentages were used. Appropriate comparison tests including the chi-square test, analysis of variance, or Kruskal-Wallis H test followed by Tukey's *post hoc* test were used for comparison among groups for categorical and continuous variables, respectively. The significance level for all statistical tests was set at 0.05, and adjusted *P* values < 0.05 in multiple comparisons. All statistical analyses were performed using SPSS 21 software (IBM, Chicago, IL). Principal component analysis (PCA) and orthogonal partial least squares discrimination analysis were performed in SIMCA-P 13.0 software (Paris, France). The Nightingale rose diagram was conducted by Apache ECharts (https://echarts.apache.org).

METABOLOMIC ANALYSIS

We performed several comparisons to find the metabolites with significantly differential expressions among groups ($P < 0.05$): (1) comparison between healthy controls and those with ALC, (2) comparison between compensated cirrhosis and those without cirrhosis (CC/NC); and (3) comparison between decompensated cirrhosis and those without cirrhosis (DC/NC). We used the following two approaches to explore the metabolites associated with the disease progression. First, the shared metabolites of the two comparisons (CC/NC and DC/NC) were identified; these were the metabolites uniquely related to ALC independence of compensatory/decompensatory states. Next, we characterized metabolites that were uniquely identified in DC/NC but not in the shared metabolites with CC/NC; these represented metabolites associated with decompensation in patients with ALC. We then annotated these selected metabolites according to the Human Metabolome Databases. Based on those annotated metabolites, the hierarchical clustering was used to develop a metabolic fingerprint consisting of a cluster of metabolites using the area under the curve (AUC) and *P* values in differentiating patients with ALC with different phenotypes, from NC and CC to DC. The phenotype-associated fingerprint metabolites were then projected to one eigenmetabolite by dimension reduction, to visually observe the difference between groups from a series of fingerprint metabolites as previously described.[14,15] The eigenmetabolite was further investigated to discover the relationships associated with demographics, baseline laboratories, and noninvasive assessment for fibrosis (the aspartate aminotransferase [AST]–to-platelet ratio index, APRI)[16] and disease severity (the Model for End-Stage Liver Disease [MELD]).[17] The alterations of the metabolic fingerprint between groups was displayed by the Nightingale rose diagram using the normalized relative intensity value of each metabolite within different groups. Finally, we combined the metabolic pathways of these phenotype-associated fingerprint metabolites to identify the underlying mechanisms associated with ALC.

Results

BASELINE DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE STUDY COHORT

The detailed demographic and baseline laboratory data are found in Table 2. Patients with ALD without cirrhosis (mean age 49 years old) and those with ALC (mean age 51 years old) were older than healthy controls (mean age 39 years old). However, there was no difference in patients' age and body mass index among those with ALD without cirrhosis compared to those with ALC. Most of the patients were male. The estimated daily alcohol consumption in patients with ALD without cirrhosis (NC), those with compensated cirrhosis (CC), and decompensated cirrhosis (DC) was 140 g, 252 g, and 224 g, respectively. Patients with ALC, as expected, had a higher serum level of total bilirubin (TBil) and AST but lower serum albumin (ALB), when compared with controls. The levels of total bile acids, prothrombin time (PT), and international normalized ratio (INR) in CC were higher than those in the NC group, and the levels of cholinesterase (CHE), triglyceride, and white blood cells in CC

TABLE 2. COMPARISON OF CHARACTERISTICS AMONG PATIENTS WITH NC, CC, AND DC

	Healthy Volunteers (n = 14)	NC (n = 16)	CC (n = 27)	DC (n = 79)	*P*	*P**	*P*†	*P*‡
Age, years	39 ± 8	49 ± 6	51 ± 8	51 ± 9	0.000	0.002	0.477	0.872
Male, n (%)	9 (64.28)	16 (100.00)	27 (100.00)	77 (97.47)	—	—	—	—
BMI, $kg·m^{-2}$	21.3 ± 0.8	23.0 (21.0, 25.6)	24.7 (22.6, 29.1)	23.8 (21.6, 26.9)	0.152	0.015	0.066	0.146
ALT, $U·L^{-1}$	29.38 ± 16.82	38.0 (21.8, 102.8)	25.0 (18.0, 35.0)	28.0 (17.0, 36.0)	0.146	0.087	0.048	0.845
AST, $U·L^{-1}$	24.0 (20.0, 29.5)	36.0 (22.5, 48.0)	40.0 (24.0, 57.0)	50.0 (32.0, 79.0)	0.000	0.053	0.792	0.041
AST/ALT	1.10 ± 0.53	1.1 ± 0.9	1.6 ± 0.7	2.0 ± 0.7	0.000	0.483	0.038	0.005
ALP, $U·L^{-1}$	64.83 ± 30.08	131.5 (96.8, 179.8)	113.0 (81.0, 162.0)	142.0 (102.0, 194.0)	0.000	0.000	0.513	0.094
GGT, $U·L^{-1}$	36.58 ± 33.26	76.0 (36.8, 210.3)	49.0 (27.0, 95.0)	61.0 (35.0,171.0)	0.014	0.011	0.187	0.249
TBil, $μmo·L^{-1}$	11.49 ± 5.17	15.39 (13.68, 57.80)	23.94 (17.10, 39.33)	39.33 (25.65, 73.53)	0.000	0.002	0.580	0.003
DBil, $μmo·L^{-1}$	4.3 (2.8, 4.9)	8.1 (4.8, 40.3)	9.8 (7.3, 20.7)	23.0 (12.4, 48.8)	0.000	0.001	0.802	0.001
TBA, $μmo·L^{-1}$	2.0 (1.0, 6.0)	10.5 (5.0, 14.0)	42.0 (11.0, 90.0)	66.0 (31.0,114.0)	0.000	0.002	0.006	0.111
ALB, $g·L^{-1}$	42.0 ± 5.7	37.5 (33.3, 40.8)	35.0 (27.0, 40.0)	28.0 (25.0, 32.0)	0.000	0.009	0.237	0.001
CHE, $U·L^{-1}$	6,695 ± 2,925	5,966 (4,615, 7,668)	4,179 (3,057, 5,110)	2,546 (1,912, 3,479)	0.000	0.137	0.004	0.000
CRE, $μmo·L^{-1}$	72.3 (13.0, 82.8)	79.5 (72.5, 83.0)	67.0 (60.0, 72.0)	70.0 (63.0, 87.0)	0.035	0.274	0.001	0.031
PT, seconds	10.8 ± 0.8	10.9 (10.3, 11.5)	12.5 (11.6, 15.2)	14.6 (12.4, 16.7)	0.000	0.018	0.000	0.016
INR, IU	0.9 ± 0.2	1.0 (0.9, 1.0)	1.1 (1.0, 1.3)	1.3 (1.1, 1.5)	0.000	0.007	0.000	0.015
TC, $mmol·L^{-1}$	2.2 ± 2.2	4.9 (3.6, 5.7)	3.7 (3.2, 4.6)	3.2 (2.6, 4.3)	0.002	0.011	0.061	0.070
TG, $mmol·L^{-1}$	0.7 ± 0.8	1.5 (1.1,2.3)	1.0 (0.8,1.3)	0.9 (0.6,1.2)	0.001	0.005	0.013	0.134
WBC, mm^3	7,113 ± 3,081	9,223 (6,333, 9,223)	4,680 (3,290, 5,860)	4,700 (2,600, 7,210)	0.000	0.086	0.001	0.538
PLT, $10^9·L^{-1}$	261.5 (205.3, 338.3)	203.5 (129.3, 277.0)	86.0 (55.0, 122.0)	82.0 (51.0, 122.0)	0.000	0.098	0.001	0.685
With ascites, %	0 (0.00)	0 (0.00)	0 (0.00)	70 (88.61)	—	—	—	—
With HE, %	0 (0.00)	0 (0.00)	0 (0.00)	4 (5.06)	—	—	—	—
Duration of drinking, years	2 (0, 4)	20 (10, 26)	20 (20, 30)	20 (20, 30)	0.114	0.000	0.240	0.341
Estimated daily alcohol intake, g	10 (0, 30)	140 (73.5, 273)	252 (140, 280)	224 (140, 280)	0.181	0.000	0.103	0.860
Abstinent, n (%)					—	0.594	0.075	—
≥1 month	—	10 (62.5)	19 (70.4)	40 (50.6)				
<1 month	—	6 (37.5)	8 (29.6)	39 (49.4)				
No abstinence	—	0 (0.0)	0 (0.0)	0 (0.0)				
APRI score	—	0.5 (0.2, 0.7)	1.0 (0.6, 2.4)	1.5 (0.8, 2.4)	0.000	—	0.019	0.115
MELD score	—	6.7 (6.4, 10.9)	9.0 (7.2, 12.7)	14.0 (10.3, 17.0)	0.000	—	0.564	0.000

Note: Data are reported as mean ± SDs or median (upper quartile, lower quartile) unless otherwise noted as n (%). *P* is the significant difference among the four groups.
*Significant difference between healthy volunteers (control cohort) and NC patients.
†Significant-difference between NC and CC patients.
‡Significant difference between CC and DC patients.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; BMI, body mass index; CRE, creatinine; DBil, direct bilirubin; GGT, gamma-glutamyl transpeptidase; HE, hepatic encephalopathy; PLT, platelets; PT, prothrombin time; TBA, total bile acid; TC, total cholesterol; TG, triglyceride; WBC, white blood cell.

were significantly lower than those in the NC group. Patients in the DC group had a significantly higher level of serum TBil, PT and INR, but lower level of ALB and CHE when compared with those in the CC group. At the time of enrollment, 88.6% and 5.1% of patients in the DC group had underlying ascites and hepatic encephalopathy, respectively. APRI score is a noninvasive marker for evaluation and staging for liver fibrosis.[16] We found that patients in the DC group had the highest APRI and MELD score compared with those in the NC and CC groups.

METABOLOMIC PROFILES IN CONTROLS AND PATIENTS WITH ALC

To test the hypothesis that patients with alcoholic cirrhosis had unique metabolomic profiles compared

with controls, we performed the PCA of serum metabolites and found the distinct separation of metabolites in controls and those with ALC (Supporting Fig. S1A). Heatmap analysis displayed differentiation of serum metabolites in healthy controls and patients with ALC (Supporting Fig. S1B).

METABOLOMIC PROFILES IN ALD PROGRESSION

To determine the metabolomic profiles associated with ALD progression, we focused our analysis of serum metabolites in patients with ALD without cirrhosis (NC), compensated cirrhosis (CC), and decompensated cirrhosis (DC). We observed significant alterations in metabolomic profiles from either positive or negative modes of mass spectrometry, in different stages of ALD from NC, CC, and DC (Fig. 1A-D and Supporting Fig. S2). When compared with NC cases, there were a total of 2,112 metabolites that were uniquely expressed in patients with cirrhosis (CC+DC). Of these, 840 metabolites (of these, 143 were annotated in the database) were found in both the CC and DC groups, suggesting that these metabolites were associated with alcoholic cirrhosis, regardless of the compensatory status. A total of 1,272 metabolites (of these, 172 were annotated in the Human Metabolome Database) were uniquely expressed only in patients with DC; this metabolic signature was likely associated with the decompensated cirrhotic state in patients with ALC. The PCA analysis was performed when we observed the distinct separation of 143 cirrhosis-related metabolites between NC and cirrhosis groups (Fig. 1F) and 172 DC-related metabolites between CC and DC groups (Fig. 1G). The full details of these metabolites are shown in Supporting Fig. S3.

SELECTION OF METABOLIC FINGERPRINT UNIQUELY EXPRESSED IN PATIENTS WITH ALCOHOLIC CIRRHOSIS

We computed the AUC and *P* values of each of the 143 cirrhosis metabolites, respectively, to identify those with significantly differential expression in patients with ALC (either CC or DC) compared to ALD without cirrhosis (NC), as shown in a hierarchical cluster and heatmap analysis. We identified the highest relevant cluster with the top 38 metabolites, which were highly expressed in patients with cirrhosis (Fig. 2 Ai). The distinct separation of these 38 cirrhosis-related metabolites was also observed between controls and those with ALC, based on the PCA analysis (Supporting Fig. S1C). The AUC for this cluster of metabolites ranged from 0.79 to 0.87 with *P* values < 0.05 (Supporting Table S1). We also found a significant difference in these 38 metabolites when the analyses were stratified by compensatory status in patients with ALC, between CC and NC, and between DC and NC (Fig. 2Aii). We next computed the eigenmetabolites of these 38 metabolites by PCA and found a significant increase in eigenmetabolites from healthy controls, NC, CC, and DC (Fig. 2B).

COMPARISONS BETWEEN THE NEWLY IDENTIFIED CIRRHOSIS-ASSOCIATED METABOLIC FINGERPRINT AND APRI TO DIFFERENTIATE THE STATUS OF FIBROSIS AND ALCOHOLIC CIRRHOSIS

Based on our findings that eigenmetabolites of these 38 metabolites progressively increases from NC to DC, we hypothesized that their levels may be associated with underlying fibrosis during disease progression. We performed a linear regression analysis and found a significant association between the eigenmetabolites of these 38 metabolites and the APRI score (r = 0.61; Fig. 2C).

SELECTION OF METABOLIC FINGERPRINT UNIQUELY EXPRESSED IN ALCOHOLIC CIRRHOSIS WITH DECOMPENSATION

We used the same approach to refine the unique metabolic fingerprint among 172 metabolites for differentially expressed metabolites associated with alcoholic cirrhosis with decompensation. We identified the relevant cluster of the top 64 metabolites with differentially expressed in patients with DC (Fig. 3A and Supporting Table S2). As expected, we also observed the distinct separation in the level of these metabolites when compared with healthy controls (Supporting Fig. S1D). We next computed an eigenmetabolite of these 64 metabolites and found a significant increase

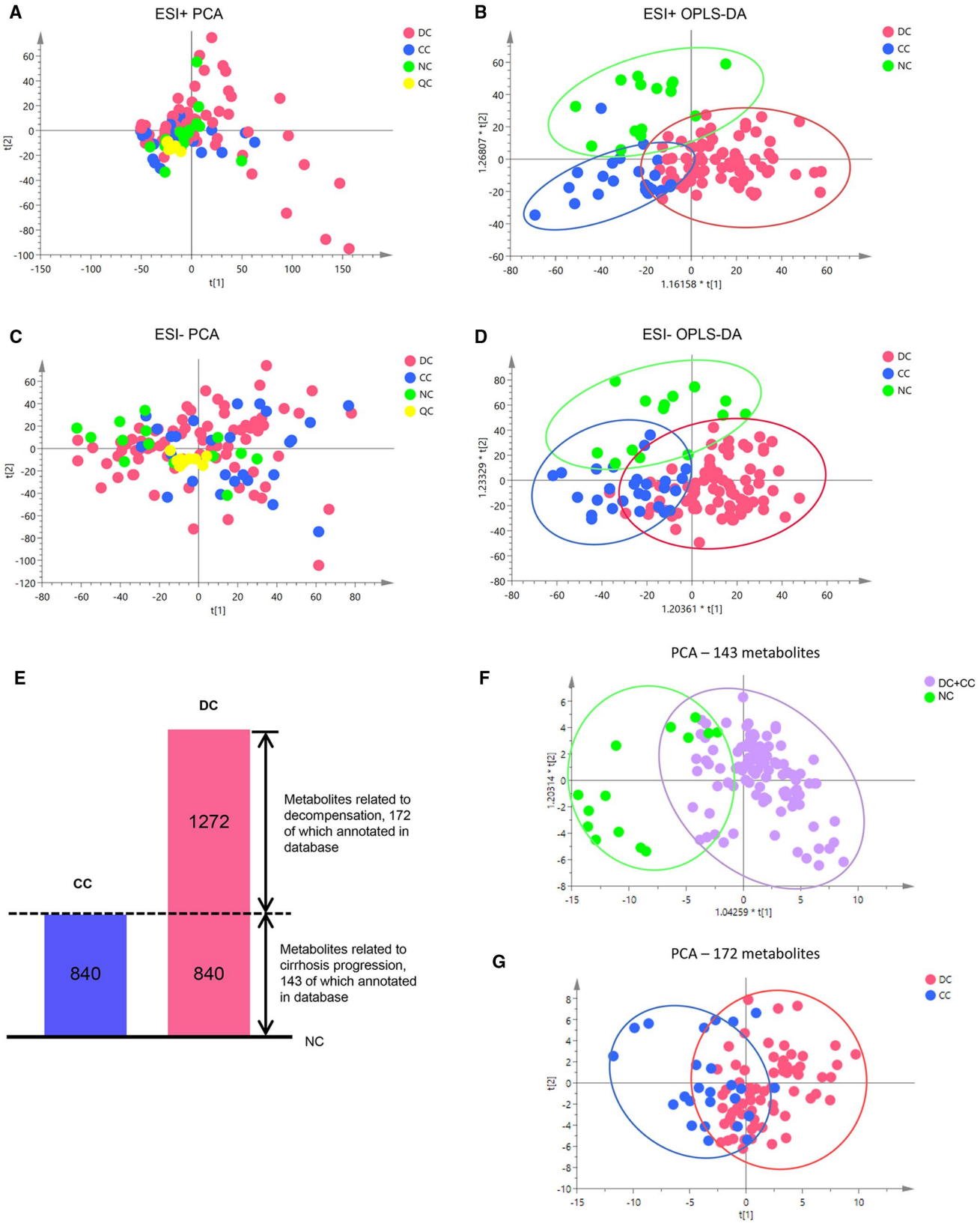
A
ESI+ PCA
DC
CC
NC
QC
t[1]
t[2]
B
ESI+ OPLS-DA
DC
CC
NC
1.16158 * t[1]
1.26807 * t[2]
C
ESI- PCA
DC
CC
NC
QC
t[1]
t[2]
D
ESI- OPLS-DA
DC
CC
NC
1.20361 * t[1]
1.23329 * t[2]
E
DC
1272
Metabolites related to decompensation, 172 of which annotated in database
CC
840
840
Metabolites related to cirrhosis progression, 143 of which annotated in database
NC
F
PCA – 143 metabolites
DC+CC
NC
1.04259 * t[1]
1.20314 * t[2]
G
PCA – 172 metabolites
DC
CC
t[1]
t[2]

FIG. 1. Metabolomic profiling in patients with ALD at different stages. (A-D) PCA and orthogonal partial least squares discrimination analysis plots with all metabolic variables among NC, CC, and DC patients under positive and negative model of mass spectrometry, respectively. (E) Metabolites with significant differences in CC or DC in comparison to NC. The shared section of the diagram indicated 840 metabolites associated with cirrhosis progression, of which 143 metabolites were annotated according to the Human Metabolome Database. There are 1,272 metabolites uniquely associated with hepatic decompensation in patients with alcoholic cirrhosis; 172 metabolites were annotated. (F) PCA plot of CC+DC versus NC with 143 annotated metabolites related to cirrhosis. (G) PCA plot of DC versus CC with 172 annotated metabolites related to hepatic decompensation. The QC samples are clustered together in the PCA plots, indicating the stability and technical reproducibility. Abbreviation: OPLS-DA, orthogonal partial least squares discrimination analysis.

in its level from healthy controls, NC, and those with ALC. Of importance, its level was significantly higher in DC compared with CC (P = 0.02; Fig. 3B) and was associated with MELD scores (r = 0.52; Fig. 3C).

USE OF METABOLIC FINGERPRINTS TO UNRAVEL UNDERLYING PATHOPHYSIOLOGICAL CHANGES DURING THE PROGRESSION OF ALD

To better our understanding of the role of these metabolites on the pathological changes during ALD progression, we conducted the Nightingale rose diagram of differentially expressed metabolites in patients with ALC stratified by metabolic pathways (Fig. 4).

Cirrhosis-Associated Metabolic Fingerprint Reveals Metabolites Associated With Lipid Metabolic Pathway

Cirrhosis-associated metabolic fingerprint revealed the metabolites in the lipid metabolic pathway, with the elevation of serum long-chain fatty acids (e.g., bovinic acid, stearic acid, vaccentic acid, palmitic acid) in patients with alcoholic cirrhosis (Fig. 4 and Supporting Table S3).

Cirrhosis-Associated Metabolic Fingerprint Reveals Metabolites Associated With Alterations in the TCA Cycle and Mitochondrial Respiratory Chain

Alcohol metabolism can lead to alterations in the redox state, leading to the inhibition of a key pathway in the TCA cycle and its intermediary metabolites.[18,19] Ubiquinones (coenzyme Q [CoQ]) is a unique electron carrier in the mitochondrial respiratory chain that is synthesized on site by a nuclear-encoded multiprotein complex. It receives electrons from different redox pathways, notably from NADH, from the TCA cycle.[19] We observed a decrease in CoQ, which was further confirmed by the inhibition of its synthesis pathway from 3-hexaprenyl-4,5-dihyd roxybenzoic acid and 2-(hydroxyamino) benzoic acid. The disturbance of intermediary metabolites (i.e., acetyl donors and lactoyl donors) led to an increase in acetyl-derived or lactoyl-derived products, such as N-acetyl-phenylalanine, N1-acetylspermidine, N-lactoylphenylalanine, or N-acetyl-S-allylcysteine (Fig. 4).

Cirrhosis-Associated Metabolic Fingerprint Reveals Metabolites Associated With Increased Proteolysis

We observed a significant increase in the markers of proteolysis and proteolysis-related metabolites (e.g., threonyl-prolyl-arginyl-lysine, an incomplete breakdown product of protein catabolism) in patients with alcoholic cirrhosis (Fig. 4 and Supporting Table S3).

Cirrhosis-Associated Metabolic Fingerprint Reveals Metabolites Associated With Bile Acid Pathway

The alterations of the metabolites in the bile acid pathway were observed in patients with alcoholic cirrhosis. We found the accumulation of lithocholic acid derivatives, chenodeoxycholic acid derivatives, and the upstream metabolite of bile acid synthesis, dihydrocortisol, in patients with alcoholic cirrhosis (Fig. 4 and Supporting Table S3).

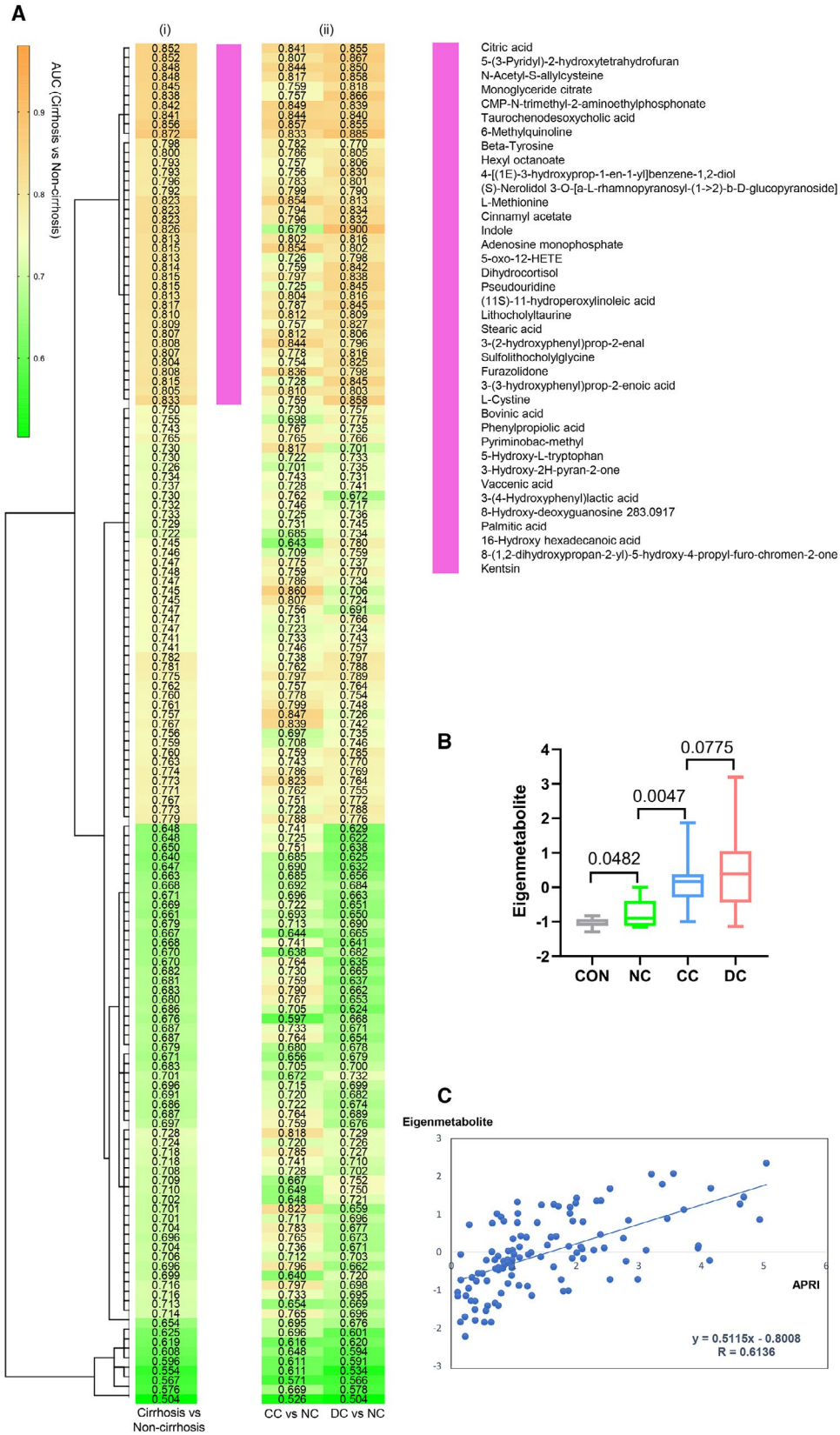
A
(i)
(ii)
AUC (Cirrhosis vs Non-cirrhosis)
0.9
0.8
0.7
0.6
Citric acid
5-(3-Pyridyl)-2-hydroxytetrahydrofuran
N-Acetyl-S-allylcysteine
Monoglyceride citrate
CMP-N-trimethyl-2-aminoethylphosphonate
Taurochenodesoxycholic acid
6-Methylquinoline
Beta-Tyrosine
Hexyl octanoate
4-[(1E)-3-hydroxyprop-1-en-1-yl]benzene-1,2-diol
(S)-Nerolidol 3-O-[a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside]
L-Methionine
Cinnamyl acetate
Indole
Adenosine monophosphate
5-oxo-12-HETE
Dihydrocortisol
Pseudouridine
(11S)-11-hydroperoxylinoleic acid
Lithocholyltaurine
Stearic acid
3-(2-hydroxyphenyl)prop-2-enal
Sulfolithocholylglycine
Furazolidone
3-(3-hydroxyphenyl)prop-2-enoic acid
L-Cystine
Bovinic acid
Phenylpropiolic acid
Pyriminobac-methyl
5-Hydroxy-L-tryptophan
3-Hydroxy-2H-pyran-2-one
Vaccenic acid
3-(4-Hydroxyphenyl)lactic acid
8-Hydroxy-deoxyguanosine 283.0917
Palmitic acid
16-Hydroxy hexadecanoic acid
8-(1,2-dihydroxypropan-2-yl)-5-hydroxy-4-propyl-furo-chromen-2-one
Kentsin
Cirrhosis vs Non-cirrhosis
CC vs NC
DC vs NC
B
Eigenmetabolite
0.0775
0.0047
0.0482
CON
NC
CC
DC
C
Eigenmetabolite
APRI
y = 0.5115x - 0.8008
R = 0.6136

FIG. 2. Selection of metabolic fingerprint uniquely expressed in patients with alcoholic cirrhosis. (A[i]) Hierarchical cluster analysis of the AUCs and *P* value assessing the discriminating accuracy of each of the 143 metabolites in differentiating cirrhosis from ALD without cirrhosis (NC). (A[ii]) The corresponding metabolites' AUC and *P* values in accessing CC or DC relative to NC. Vertical violet bar identified the 38-metabolite cluster highly associated with cirrhosis, the cirrhosis-associated metabolite fingerprint. (B) The increasing trend of the eigenmetabolite (38 fingerprint metabolites) from controls and patients with different stages of ALD progression. (C) Relationship between the cirrhosis-associated eigenmetabolite and APRI values. Abbreviation: Con, controls.

Cirrhosis-Associated Metabolic Fingerprint Reveals Metabolites Associated With Alterations in the Oxidative Stress Pathway

We observed a significant increase in oxidative stress products, 8-(1,2-dihydroxypropan-2-yl)-5-hydroxy-4-propyl-2H,8H,9H-furo[2,3-h]chromen-2-one and 5-(3-pyridyl)-2-hydroxytetrahydrofuran, in patients with alcoholic cirrhosis. Additionally, as a consequence of oxidative stress, a DNA damage marker, 8-hydroxy-deoxyguanosine, was also significantly increased in patients with alcoholic cirrhosis.

Discussion

In this study, we performed an untargeted serum metabolomic analysis on patients with ALD with different stages of the disease process, and compared them with from those without clinically apparent cirrhosis, compensated, and decompensated cirrhosis. We identified a unique panel of 38 metabolites uniquely expressed in patients with alcoholic cirrhosis when compared to ALD without cirrhosis and healthy controls. The eigenmetabolite of these metabolites is correlated with a noninvasive marker for fibrosis, the APRI score, and has a better diagnostic performance than that of the APRI to discern patients with alcoholic cirrhosis. We also found a metabolic fingerprint of 64 metabolites with unique expression in patients with alcoholic cirrhosis with decompensation and healthy controls. The eigenmetabolite of these metabolites is significantly associated with the MELD score. Collectively, these metabolites represent the changes in several metabolic pathways that occur during the progression from ALD without cirrhosis, compensated, and decompensated cirrhosis.

Once ingested, alcohol metabolism can serve as the energy source, generating approximately 7 kcal per gram of alcohol consumed. In excessive alcohol users, the energy generated by alcohol intake provides more than half of the total daily calories,[20] causing a significant shift in cellular energy supply, notably in hepatocytes. The accumulation of acetaldehyde, the by-product of alcohol metabolism, causes the disturbance in intrahepatic lipid metabolism by shifting fatty acid oxidation toward fatty acid synthesis.[8,11,21] The inhibition of fatty acid oxidation results in an increase in circulating free fatty acids,[22] as observed in our metabolic fingerprint with the dysregulation of metabolites in lipid metabolism pathway in patients with alcoholic cirrhosis (Fig. 4). The changes in the redox state with an increase in the $NADH/NAD^+$ ratio also lead to the inhibition of two enzymes in the citric acid cycle: isocitrate dehydrogenase-mediated isocitrate decarboxylation and α-ketoglutarate (α-KG) dehydrogenase complex-mediated α-KG decarboxylation.[18,23] Patients with alcoholic cirrhosis have the alterations in the level of CoQ, the mitochondrial respiratory chain carrier and a potent membrane anti-oxidant.[19,24] The observation may be related to the changes in the inflammatory cytokines, leading to the depletion of CoQ in these patients.[25,26] Alcohol metabolism causes the accumulation of ROS products and intracellular oxidative stress.[27,28] As an antioxidant, the reduction of CoQ during the progression of compensated to decompensated state in patients with alcoholic cirrhosis may perpetuate the ROS generation and worsen liver injury, as indicated by an increase of metabolites in the oxidative stress pathway in our study.[29,30]

The alterations in serum amino acid have been described in patients with ALD and confirmed in our study (Fig. 4).[11,31] A previous study reported a decrease in serum level of branched-chain amino acids (BCAAs) along with the α-ketoacids produced by BCAA transaminase in patients with alcoholic cirrhosis.[11] The level of amino acids is influenced by dietary protein deficiency, alcohol consumption, and the stage of ALD.[31] We also observed an increase in the level of methionine in patients with alcoholic cirrhosis (Fig. 4). Methionine

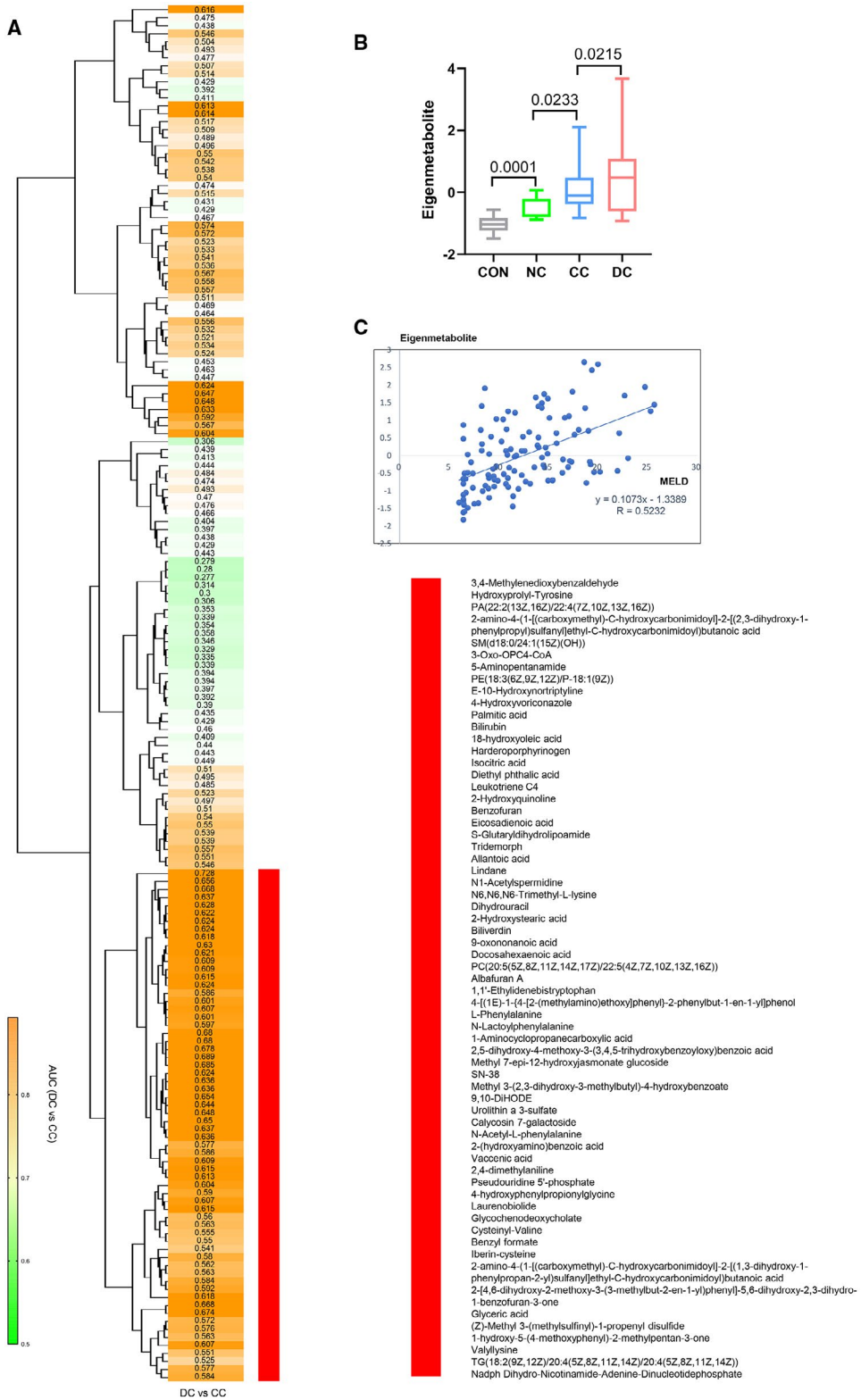

FIG. 3. Selection of metabolic fingerprint uniquely expressed in alcoholic cirrhosis with decompensation. (A) Hierarchical cluster analysis of the AUCs and *P* value assessing the discriminating accuracy of each of the 172 metabolites in differentiating DC from CC. Vertical red bar identified the 64-metabolite cluster highly associated with DC, the decompensation-associated metabolite fingerprint. (B) Increasing trend of the eigenmetabolite (64 fingerprint metabolites) from controls and patients with different stages of ALD progression. (C) Relationship between the decompensation-associated eigenmetabolite and MELD score.

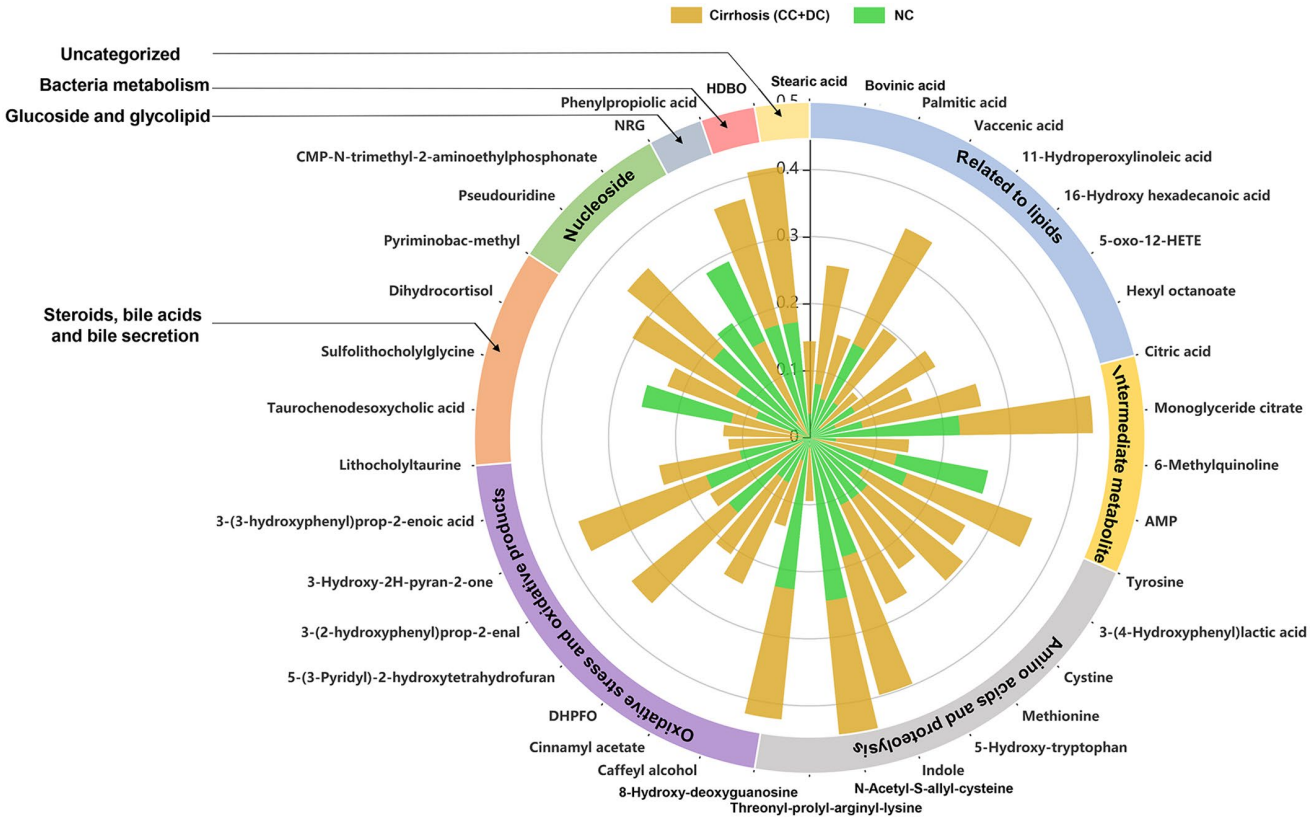

FIG. 4. Use of metabolic fingerprints to unravel underlying pathophysiological changes during the progression of ALD. Nightingale rose diagram of cirrhosis-related metabolic fingerprint. The value of relative intensity of each metabolite was normalized within the two groups. The whole set of metabolites in the fingerprint was classified into different metabolic pathways, including lipids, intermediate metabolism, amino acids and proteolysis, oxidative stress and oxidative products, steroid, bile acids and bile secretion, nucleoside, pentose phosphate pathway, glucoside and glycolipid, bacteria metabolism, and uncategorized. Abbreviations: AMP, adenosine monophosphate; DHPFO, 8-(1,2-dihydroxypropan-2-yl)-5-hydroxy-4-propyl-2H,8H,9H-furo[2,3-h]chromen-2-one; HDBO, 2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one; NRG, (S)-nerolidol 3-O-(a-L-rhamnopyranosyl-[1->2]-b-D-glucopyranoside).

adenosyltransferase (MAT) is a key enzyme that converts methionine into S-adenosylmethionine (SAMe), the principal biological methyl donor made in all mammalian cells.[32,33] Patients with ALD have a reduction in hepatic MAT1A activity, resulting in the reduction of hepatic SAMe levels, the product of methionine metabolism.[34,35] The interruption in the vicious feed-forward cycle of low MAT1A and SAMe also affects liver nonparenchymal cells. SAMe inhibits hepatic stellate cell activation[36] and lipopolysaccharide-induced inflammatory cytokine production in macrophages.[37] Taken together, the dysregulation of the methionine metabolism plays an important role in ALD disease progression. Among the complications of liver disease, sarcopenia, or muscle loss with contractile dysfunction, occurs most frequently and is most severe in patients with alcoholic cirrhosis.[38] Ethanol metabolism in the muscle contributes to decreased protein synthesis and increased autophagy proteolysis.[39-41] Our metabolomics data showed an increase in the level of proteolysis markers, especially in those with DC.

The dysregulation of bile acid homeostasis has been described in patients with alcoholic cirrhosis,[11] and we observed changes in the level of bile acids during the disease progression. The regulation of bile acid required energy-dependent bile acid transporters—a process that may be affected due to the ineffective oxidative phosphorylation to generate an adenosine triphosphate (ATP) secondary to low CoQ.[42] The accumulation of lithocholyltaurine (Fig. 4) may lead to hepatocyte apoptosis and worsening liver function during disease progression.[43]

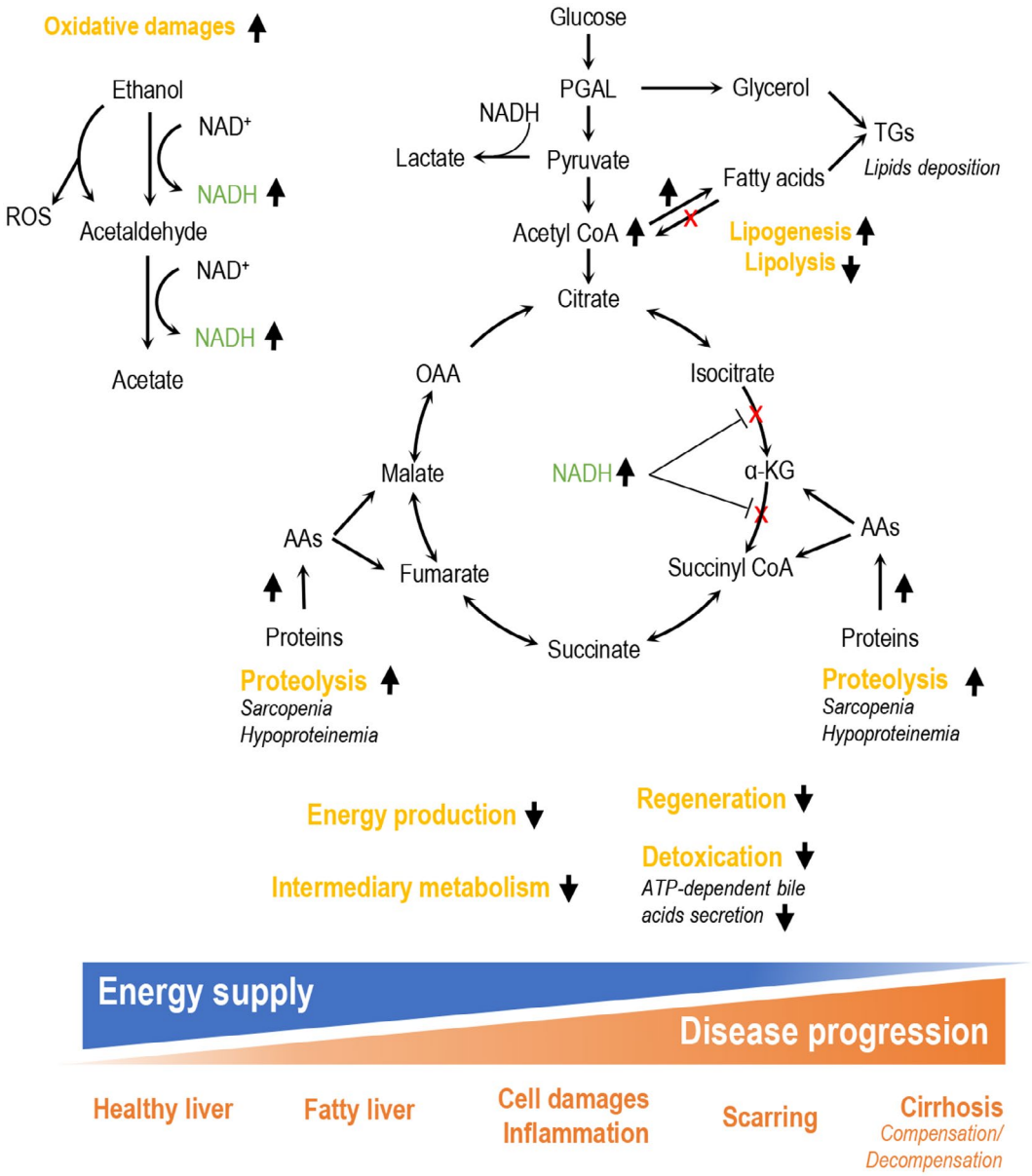

FIG. 5. Summary of metabolic pathways associated with ALD progression. Our data suggested the dysregulation in cellular energy supply pathway during ALD progression. The high NADH/NAD$^+$ ratio, as a consequence of alcohol metabolism, inhibits the TCA cycle by inhibiting two irreversible enzymic reactions: isocitrate dehydrogenase–mediated isocitrate decarboxylation and α-KG dehydrogenase complex–mediated α-KG decarboxylation. As a result, this leads to the suppression of intermediary metabolites, fatty acid oxidation, carbohydrate catabolism, and the reduction of energy supply (ATP) from nutrients. The energy supply disturbance may interfere with bile acid secretion, an ATP-dependent process through bile acid transporters. An increase in the level of proteolysis markers is also observed during the disease progression. Abbreviations: AA, amino acid; CoA, coenzyme A; OAA, oxaloacetate; TG, triglyceride.

The summary of metabolic pathways associated with ALD progression is shown in Fig. 5. Our data suggest the dysregulation in cellular energy supply pathway during disease progression. The alteration in serum metabolites reflects an activation of oxidative stress and proteolysis during the course of the disease, from compensated to decompensated alcoholic cirrhosis. The changes in serum metabolomic phenotypes in our study appear to mimic those in the liver. A study of integrated hepatic metabolomic and transcriptomic analyses of patients with alcoholic hepatitis, a severe form of ALD, demonstrated specific changes in the

levels of intermediates of glycolysis/gluconeogenesis and the TCA cycle, suggesting an extensive reprogramming of glucose metabolism in these patients.[44] Future studies to further explore the clinical applicability of these metabolites as biomarkers of disease progression or therapeutic targets in patients with ALD should be explored.

Our study has several strengths. We used comprehensive metabolic profiling and identified the metabolic fingerprint associated with the disease progression in a large cohort of patients with different stages of ALD using well-defined diagnostic criteria (Table 1). Our analysis provides information to further our understanding of the pathological changes of several metabolic pathways, based on serum metabolites, during the progression of ALD. A panel of a metabolic fingerprint is closely associated with the APRI score and has a better diagnostic ability in discerning fibrosis status. A unique metabolic fingerprint is also associated with the decompensatory state in patients with alcoholic cirrhosis and MELD score. However, we acknowledge several limitations. First, we used self-reporting questionnaires to determine the quantity and duration of drinking to determine cases with ALD; the recall methods influence its accuracy. Second, we used the intersubject study design to determine the metabolites uniquely expressed during the disease progression. Although we are able to obtain valuable information, using the intrasubject data by following the patient prospectively from the compensated to decompensated stage would be the ideal approach, although time-prohibitive, to identify the unique metabolites associated with disease progression. Third, our study cohort is of Asian origin, a population with known genetic polymorphisms in alcohol metabolizing enzymes that markedly affect alcohol metabolism.[8] Whether the observed changes in serum metabolites during the disease progression are similar to those in Caucasians is not known, and it deserves additional study. Finally, a gender difference in alcohol pharmacokinetics and metabolism underlies an increased susceptibility of ALD in women. Future investigation to determine whether the metabolomic fingerprint differs in woman compared with men should be explored, given that almost all patients in our study were men.

In conclusion, our metabolomic analysis reveals a unique metabolomic fingerprint associated with the disturbance in several metabolic pathways during the progression of ALD (Fig. 5). Future studies are needed to validate our results, notably in a women and Caucasian cohort, to determine the practicality of the metabolic panel as the diagnostic tool for fibrosis, and to determine the prognostic significance of our metabolic fingerprint on the long-term outcomes of patients with ALD.

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