Research Article

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The Metabolic Status In Non-Alcoholic Fatty Liver Disease And Its Subtypes: A Pilot Study

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1. Abstract

1.1 Aim: To unravel novel circulating metabolites relevant to non-alcoholic fatty liver disease (NAFLD) and its subtypes, lean NAFLD (LN) and overweight/obese NAFLD (ON).

1.2 Methods: A case-control study was undertaken involving 72 newly onset NAFLD and 72 sex-, age-matched non-NAFLD inKailuan cohort. Untargeted liquid chromatography-tandem mass spectrometry was performed to detect serum metabolomics. Significantly altered metabolites were selected. Logistic regressions were used to validate the associations between candidate metabolites and diseases.

1.3 Results: Compared to non-NAFLD, the glycerophospholipid metabolism pathway was evidently changed in NAFLD, LN, and ON. Panels containing seven, sixteen, and four specific glycerophospholipids were found to discriminate NAFLD, LN, and ON. The glycerophospholipid metabolism pathway was also reprogrammed in LN vs. ON. There was a positive correlation between choline and LN [odds ratio(OR)=4.35, 95% confidence interval(95% CI): 1.36-13.90]. A panel containing choline, PC, and LPC had an AUC of 0.73 to distinguish LN from ON. Moreover, a positive relationship was discovered between LPC(20:3(8Z,11Z,14Z)) and very-low-density lipoprotein (OR=3.13, 95% CI: 1.20-8.19).

1.4 Conclusions: The reprogramming of the glycerophospholipid metabolism pathway may be principal in NAFLD and its subtypes.The disruption of PC synthesis and subsequent dysfunctional very-low-density lipoprotein secretion may be responsible for the development of LN.

2. Keywords:
Non-alcoholic fatty liver disease; Lean non-alcoholic fatty liver disease; Untargeted metabolomics; Choline; Glycerophospholipid; Very-low-density lipoprotein Shouling Wu,

3. Introduction

Non-alcoholic fatty liver disease (NAFLD) is rapidly becoming one of the most common liver diseases and often increases intra- and extra-hepatic disease risks[1-3]. Despite its importance in clinical practice, there is a lack of easily accessible biomarkers for early diagnosis[4]. Metabolomics may be helpful for research of NAFLD,a disease with a dynamic and complex phenotype, resulting from the multiple-level interactions between genetic and environmental factors[5-7]. Potential circulating metabolites and several metabolic pathways in NAFLD have been found by means of metabolomics, such as variations in amino acid metabolism and key aspects of lipid metabolism, including fatty acids, triglycerides, bile acids, and phospholipids[8, 9]. However, the findings are inconclusive and inconsistent. Further investigations are needed to gain insights into other new early biomarkers and principal metabolic pathways influenced by NAFLD. Notably, a subset of patients with NAFLDis lean[10]. Growing evidence implicates that lean NAFLD (LN) may be a distinct entity with respect to pathophysiological mechanism. Several cohort studies have reported a higher all-cause[11, 12], liver-related mortality, and higher occurrence ofsevere liver disease[13, 14] in LN patients than their counterparts with overweight/obese NAFLD (ON). Additional evidence from recent metabolomics studies also showed a distinctive metabolite profile in LN from ON, indicating different metabolic reprogramming and adaptation between LN and ON. Results from targeted metabolomics studies in Caucasians revealed changes in circulating phospholipids, including phosphatidylcholines (PCs), lysophosphatidylcholines (LPCs), amino acids[15], and bile acids[16] in LN compared to ON. A study in the Chinese population reported distorted metabolism characterized by disordered fatty acid and amino acid profiles in LN and ON patients in comparison to non-NAFLD[17], but they failed to compare LN and ON directly. More studies based on metabolomics are needed to unravel the unique mechanisms of LN and develop subtype-specific markers for clinical applications, particularly in Chinese population. Therefore, employing a case-control design nested within the Kailuan cohort, this

pilot study aimed to identify metabolic markers of NAFLD, and to reveal the metabolic discrepancies between LN and ON. Our results will hopefully provide insightful evidence for establishing diagnosis biomarkers and developing clinically relevant targets for pharmacological interventions of NAFLD and its subtypes.

4. Methods

4.1 Study design and participants

This study adopted a cohort-based case-control design. The Kailuan cohort (Chinese Clinical Trial Registry number: ChiCTR-TNRC-11001489) is a prospective community cohort built in Kailuan of Tangshan City, Hebei Province, China, to evaluate non-communicable diseases in Chinese population. The cohort recruited 101,510 research objects who finished a baseline investigation in 2006-2007 and underwent a regular biennial follow-up in 11 local hospitals. We included 72 cases with incident NAFLD from September 1, 2017, to December 31, 2017. We defined incident NAFLD as those newly diagnosed with fatty liverusing abdominal ultrasound imaging (HD-15; Philips, Netherlands) afterexcludingsecondary causes of hepatic lipid accumulation, including viral hepatitis and excessive drinking [18]. We also selected 72 controls without NAFLD who were 1:1 matched to cases by age (±3 years) and sex during the same follow-up period. All the controls were excluded from NAFLD using the same diagnostic criteria as cases. The study was carried out in obeyance of the tenets of the Declaration of Helsinki. Ethical approval was obtained from both the Ethics Committee of the Kailuan General Hospital and the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences(2016-IFMS-006). Additionally, written informed consent was retrievedfrom all participants.

4.2 Covariates

Sociodemographic characteristics were collected by questionnaire investigation. E Height, weight, waist circumference, systolicand diastolic pressure were also measured. BMI was calculated as body weight divided by the square of height (kg/m2). Total serum cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), fasting blood glucose (FBG), alanine transaminase, c-reactive protein were quantified using an auto-analyzer (Hitachi 747; Hitachi, Tokyo, Japan) at the central lab in Kailuan General Hospital.VLDL was estimated as TC minus LDL-C and HDL-C[19]. Two insulin resistance-related indexes, including triglyceride glucose index (TyG index) and TG/HDL-C ratio[19], were also computed,among which, the TyG index was denoted as ln[TG(mmol/L)*FBG (mmol/L)/2]. Serum hepatitis B surface antigen was tested by enzyme-linked immunosorbent assay (Shanghai Kehua Bio-Engineering, KHB).

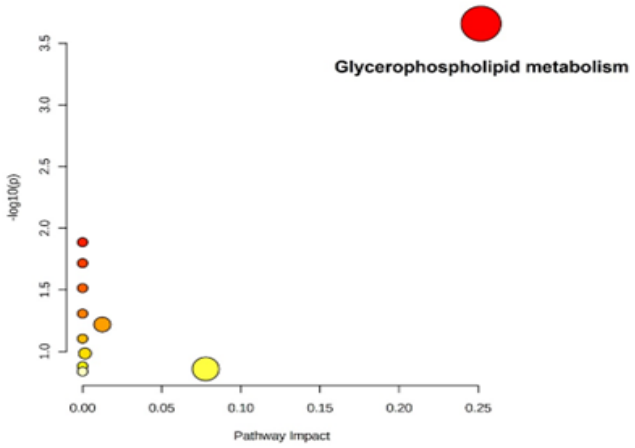
4.3 Metabolomics profiling

The serum metabolomic profiling was examined using high-throughput untargeted liquid chromatography (LC)-tandem mass spectrometry (MS) system, the reproducibility and reliability of which were assessed by quality control (QC) samples.

4.4. Serum sample preparation

100μL of serum was poured into 10μL of L-2-chlorophenylalanine, and the mixture was vortexed for 10 sec. Subsequently, a 300μL mix of methanol and acetonitrile (2/1, v/v) was added. After vortex mixing for 1 min, the extract was centrifuged at a speed of 13,000 rpm, 4℃ for 10 min. 300μL of supernatant was dried in a glass vial. Then, a 400μL mixture of methanol and water (1/4, v/v) was added and vortexed for 30 sec. The tube was then centrifuged again at 13,000 rpm, 4℃, for 10 min, after which 150μL of supernatant was filtered through 0.22μm micro-filters and relocated to an LC vial. The vial was reserved at -80℃ tillfurther LC-MSoperation. A total of 14 QC samples were formulated as a pooled sample by mixing aliquots of all samples and used to balance the LC-MS system.

Figures 1 (A) NAFLD vs. non-NAFLD



4.5. LC-MS/MS analysis

ADionex Ultimate 3000 ultra-high pressure LC system equipped with Q Exactive Plus Hybrid Quadrupole-Orbitrap MSinstalled with a heated electrospray ionization source was used to characterize the metabolite landscape in both electrospray ionization positive and negative ion modes, in which an ACQUITY UPLC HSS T3 (1.8μm, 100nm×2.1mm) was employed. The binary solvent gradient elution composed of solvent A (0.1%, v/v, formic acid dissolved in water) and solvent B (0.1%, v/v, formic acid dissolved in acetonitrile). Separation was completed by the following gradient: 0 min, 5% B; 2 min, 5% B; 4 min, 25% B; 8 min, 50% B; 10 min, 80% B; 14 min, 100% B; 15 min, 100% B; 15.1 min, 5% B; 16 min, 5% B. The injection volume was at 2μL each time. We injected QC samples every ten samples throughout the analytical run. The detailed MS conditions was showed in Supplementary Table 4.6. Raw data preprocessing and metabolite identification. The acquired raw data were preprocessed and analyzed by Progenesis QI software v2.3 (Nonlinear Dynamics, Newcastle, UK) through baseline filtering, peak identification, peak integration, retention time correction, peak alignment, and normalization. In addition, metabolites were annotated on the basis ofprecise mass, secondary fragments, and isotope distribution by mapping on the Human Metabolome Database (HMDB), the Lipid Metabolites and

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Pathways Strategy (LIPID MAPS) v2.3, Metabolite Link (METLIN), and a self-built library. The extracted data included mass-to-charge ratio (m/z), peak RT andintensities, and the RT-m/z pair was linked as the label for each ion. Ion peaks with a relative standard deviation >40% were deleted. The ion peaks with >50% missing values were also deleted, and missing values ≤50% were displacedby half of the minimal level. The quality of compound annotation was ensured by scoring. If the score was less than 36 (a total score of 60), the identification results were considered incorrect and would be deleted. In the end, data from both positive and negative ion modes were combined to form a matrix containing all the signals extracted from the raw data.

4.7 Statistical analyses

Baseline characteristics weredemonstrated as median (interquartile range) or mean ± standard deviation for continuous variables and frequency (percentage) for categorical variables, which werecompared between groups by Student's t-test or Mann-Whitney U test for continuous variables and Pearson chi-square test or Fisher exact probability method for categorical variables.SAS software, version 9.4 (SAS Institute, Cary, NC), was utilized, with two-sided P< 0.05 considered as statistically significant. We further categorized the NAFLD patients into two groups: LN (BMI <23 kg/m2) and ON (BMI≥23 kg/m2) considering BMI ≥ 23.0 kg/m2 is the cut-off recommended for the diagnosis of overweight in Asian adults[20]. Three subjects without data on BMI in 2016-2017 were excluded, and 20 LN and 49 ON were finally included. Orthogonal partial least squares discriminant analysis (OPLS-DA) after Pareto scaling was carried out to visualize the metabolite landscapeof different groups, including NAFLD vs. non-NAFLD, LN vs. non-NAFLD, ON vs. non-NAFLD, and LN vs. ON. The response permutation testing (permutation number=200) was operated to validate the robustness of the OPLS-DA model. Variable importance in projection (VIP) ranked the relative contribution of each metabolite to the OPLS-DA model. Candidate metabolites were selected referred to VIP values of the OPLS-DA model (VIP>1) together with the P value of analysis of covariance adjusted for age and sex on the log-transformed normalized peak areas (P<0.05). Differential metabolites were enriched by Homo sapiens (Kyoto Encyclopedia of Genes and Genomes) library in MetaboAnalyst 5.0 to identify significantly altered metabolic pathways. We visualized the selected metabolites in heat maps to show differences between groups using MetaboAnalyst 5.0. Logistic regressions were also used to estimate odds ratios (ORs) and 95% confidence intervals (95% CIs) to further validate the relationships between candidate metabolites (divided into two groups using the median of relative abundance as a cut-off point) and diseases in NAFLD vs. non-NAFLD,LN vs. non-NAFLD, ON vs. non-NAFLD, and LN vs. ON. A panel of significant candidate metabolites selected by logistic regression in each pairwise comparison was used to discriminate two states,and the area under the curve (AUC) was calculated.

5. Results

5.1 Baseline characteristics of NAFLD cases and non-NAFLD controls

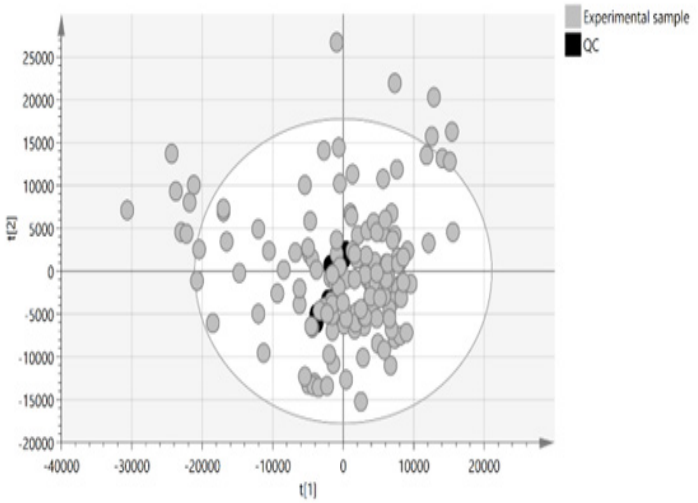
The baseline characteristics of the NAFLD patientsand healthy controls were depicted in Table 1. The NAFLD cases tended to have higher blood concentrations of TG, LDL-C, TG/HDL-C ratio, TyG index, and lower blood concentrations of HDL-C (P<0.05) than healthy controls. Noapparent differences were observed between two groups in age, sex, BMI, waist circumference, SBP, DBP, TC, FBG, c-reactive protein, and alanine transaminase (P>0.05).

Supplementary Table 1. Mass spectrometry conditions

Parameters	Positive ion	Negative ion
The mass range (m/z)	100-1,000	100-1,000
Full mass spectrometry resolution	70,000	70,000
Mass spectrometry / mass spectrometry resolution	17,500	17,500
The Collision energy (eV)	10, 20, 40	10, 20, 40
Spray voltage (V)	3800	3000
Sheath gas flow rate (Arb)	35	35
Auxiliary gas flow rate (Arb)	8	8
Capillary temperature (°C)	320	320
Aux gas heater temperature (°C)	350	350
S-lens RF level	50	50

5.2 Metabolite comparison NAFLD vs.non-NAFLD

The metabolite landscapes of the NAFLD cases and non-NAFLD controls were further deciphered from the 6132 annotated metabolites(3614 and 2518 detected from positive and negative ion mode, respectively). The QC samples were tightly clusteredin the score plot of unsupervised principal component analysis (Supplementary Figure 1), implicating the LC-MS system's good repeatability, reliability, and stability. The 400-times response permutation testing demonstrated R2Y and Q2Y values of 0.165 and -0.187, respectively (Supplementary Figure 2). The grey regression line of Q2 value with Y-axis intersect below zero validated the OPLS-DA model.



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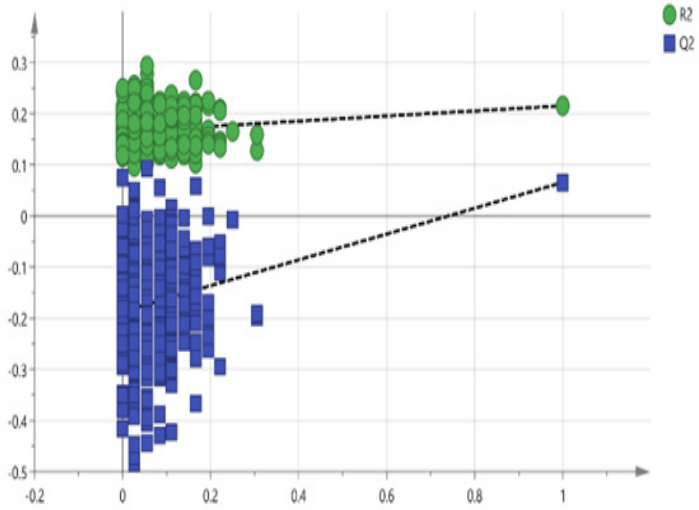
Supplementary Figure 1. Principal component analysis of quality control samples to assess the reliability of the system.
Abbreviations: QC, quality control.



Supplementary Figure 2. Response permutation testing (400 times) of the prediction model by orthogonal partial least-squares-discriminant in NAFLD vs. non-NAFLD.

The Q2 value represents the predictability of the model. The R2Y value represents the goodness of fit of the model. The grey regression line of Q2 value with Y-axis intersect below zero suggests the validity of the orthogonal partial least-squares-discriminant analysis model.
Abbreviations: NAFLD, non-alcoholic fatty liver disease.

Overall, 77 differential metabolites were screened out according to the rule (VIP>1 and P<0.05), and among them, 48 are lipids and lipid-like molecules. Glycerophospholipids covering PC (n=11), LPC (n=3), phosphatidylethanolamine (PE) (n=5), phosphatidylserine (PS) (n=2), phosphatidylinositol (PI) (n=2), phosphatidic acid (PA) (n=1), and lysophosphatidic acid (LPA)(n=1) accounted for 52.1% (25/48) of the total lipids and lipid-like molecules found (Supplementary Table 2-1, Figure 2).Pathway analysis showed a significantly enriched pathway involvingglycerophospholipid metabolism (FDR P=0.018; pathway impact 0.252; Figure 1A).We further validated the associations between differential metabolites in the glycerophospholipid pathway and NAFLD. Specific PCs (n=6) and PE (n=1) were found to be associated with NAFLD (Supplementary Table 3). A panel consisting of the above seven metabolites reached an AUC of 0.73 in discriminating NAFLD from non-NAFLD (Table 2).

5.3 Metabolite comparison LN vs. non-NAFLD and ON vs. non-NAFLD

To further identify candidate metabolites with potential to make a distinction between subtypes of NAFLD (LN and ON), two sub-comparisons were performed (LN vs. non-NAFLD and ON vs. non-NAFLD). Supplementary Table 1 showed the characteristics of subgroups. The TG, LDL-C, HDL-C, TG/HDL-C ratio, and TyG index of the LN group were between those of the ON and non-NAFLD group. In comparison between LN and non-NAFLD, 70 differential metabolites were identified, with 58 of them belonging to the categoryof lipids and lipid-like molecules (Supplementary Table 2-2).

Among the 58 lipids and lipid-like molecules, 40 were glycerophospholipids. Furthermore, pathway analysis showed a significantly enriched pathway involving glycerophospholipid metabolism (FDR P=0.032; pathway impact 0.138) (Figure 1B).Choline, PC (n=21), LPC (n=8), PE (n=6), lysophosphatidylethanolamine (LPE) (n=1), PS (n=1),and phosphatidylinositol (PI) (n=2)were identified in the glycerophospholipid metabolism pathway (Figure 2).Logistic regression revealed choline andspecific PCs (n=9), LPCs (n=2), PE (n=1), PIs (n=2), and PS (n=1) to be associated with LN. A panel of the above 16 metabolites achieved an AUC of 0.87 in discriminating LN from non-NAFLD (Table 2).

Supplementary Table 2-1. Differential metabolites in NAFLD vs. non-NAFLD.

ID	Retention time (min)	m/z	Error (ppm)	Scan mode	Postulatedidentity	Super class	Class	Sub class	Molecule composition
0.73_115.0635n	0.733067	116.0707	1.217482	Pos	L-Proline	Organic acids and derivatives	Carboxylic acids and derivatives	Aminoacids, peptides, and analogues	C5H9NO2
12.35_549.3788n	12.35067	550.3861	-1.15616	Pos	LysoPC(20:1(11Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H56NO7P
13.30_551.3953n	13.2991	552.4026	0.35191	Pos	LysoPC(20:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H58NO7P
12.00_297.2419m/z	12.00175	297.2419	-1.68835	Pos	12R-hydroxy-9Z,15Z-octadecadienoic acid	Unclassified	Unclassified	Unclassified	C18H32O3
14.29_780.5505m/z	14.28892	780.5505	-1.16765	Pos	PC(16:0/18:2(11Z,13Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C42H80NO8P

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11.02_564.3309m/z	11.02308	564.3309	0.41043	Neg	2-linoleoyl-sn-glycero-3-phosphocholine	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H50NO7P
1.36_132.1018m/z	1.3554	132.1018	-0.59934	Pos	L-Isoleucine	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues	C6H13NO2
11.04_588.3309m/z	11.03847	588.3309	0.438121	Neg	LysoPC(20:4(8Z,11Z,14Z,17Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H50NO7P
11.54_566.3467m/z	11.54315	566.3467	0.687278	Neg	PC(18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H52NO7P
11.76_554.3465m/z	11.76197	554.3465	0.382928	Neg	PC(O-16:0/1:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C25H52NO7P
4.48_368.2273m/z	4.477317	368.2273	-1.57732	Pos	(1R,2R,4S)-p-Menthane-1,2,8-triol 8-glucoside	Organic oxygen compounds	Organooxygen compounds	Carbohydrates and carbohydrateconjugates	C16H30O8
8.25_390.1905m/z	8.252417	390.1905	-1.75623	Pos	1-hydroxy-2-[6-(2-methylbut-3-en-2-yl)-7-oxo-2H,3H,7H-furo[3,2-g]chromen-2-yl]propan-2-yl acetate	Phenylpropanoids and polyketides	Coumarins and derivatives	Furanocoumarins	C21H24O6
12.46_594.3783m/z	12.46217	594.3783	1.174066	Neg	PC(20:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H56NO7P
4.77_326.1935n	4.772317	344.2273	-1.70124	Pos	Heptaethylene glycol	Organic oxygen compounds	Organooxygen compounds	Ethers	C14H30O8
4.93_370.2197n	4.9328	388.2535	-1.61354	Pos	Octaethylene glycol	Organic oxygen compounds	Organooxygen compounds	Ethers	C16H34O9
4.58_282.1673n	4.576767	300.2011	-1.89426	Pos	Hexaethylene glycol	Organic oxygen compounds	Organooxygen compounds	Ethers	C12H26O7
11.80_552.3679m/z	11.79572	552.3679	1.543731	Neg	PC(O-18:1(11Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H54NO6P
10.43_460.2687m/z	10.43192	460.2687	-1.59013	Pos	Pectachol	Phenylpropanoids and polyketides	Coumarins and derivatives	Unclassified	C26H34O6
5.53_764.4625m/z	5.529417	764.4625	-0.2516	Pos	PE-NMe2(15:0/18:4(6Z,9Z,12Z,15Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C40H72NO8P
14.25_433.2595m/z	14.25007	433.2595	-0.07154	Neg	3alpha-Hydroxy-12-oxo-5beta-chol-7-en-24-oic Acid	Unclassified	Unclassified	Unclassified	C24H36O4
12.52_340.2840m/z	12.5201	340.284	-1.83948	Pos	(-)-2,7-Dolabelladiene-6beta,10alpha,18-triol	Lipids and lipid-like molecules	Prenol lipids	Isoprenoids	C20H34O3
3.24_232.1539m/z	3.243083	232.1539	-1.74899	Pos	Butyrylcarnitine	Lipids and lipid-like molecules	Fatty Acyls	Fatty acid esters	C11H21NO4
1.08_132.0244n	1.082017	150.0581	-0.87235	Pos	THTC	Unclassified	Unclassified	Unclassified	C5H8O2S
5.57_724.4677m/z	5.573817	724.4677	-0.18478	Pos	PE(P-16:0/17:2(9Z,12Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C38H72NO7P
5.60_808.4885m/z	5.602483	808.4885	-0.52853	Pos	PS(O-18:0/18:4(6Z,9Z,12Z,15Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoserines	C42H76NO9P

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4.85_245.1161n	4.854033	246.1233	-1.457	Pos	OR-1896	Benzenoids	Benzene and substituted derivatives	Anilides	C13H15N3O2
2.14_148.0523n	2.14475	166.086	-0.57959	Pos	3,4-Dihydro-2H-1-benzopyran-2-one	Phenylpropanoids and polyketides	3,4-dihydrocoumarins	Unclassified	C9H8O2
13.49_785.5901n	13.49005	808.5817	-4.2676	Pos	PC(16:1(9Z)/20:1(11Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C44H84NO8P
13.50_781.5594n	13.50448	804.5486	-3.49457	Pos	PC(16:0/20:4(5Z,8Z,11Z,14Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C44H80NO8P
13.70_705.5886m/z	13.69787	705.5886	-2.63578	Pos	SM(d18:0/16:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C39H81N2O6P
0.71_114.0663m/z	0.705367	114.0663	1.18138	Pos	Creatinine	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues	C4H7N3O
14.68_284.2943m/z	14.67687	284.2943	-1.85076	Pos	2E-Octadecenal	Lipids and lipid-like molecules	Fatty Acyls	Fatty aldehydes	C18H34O
14.22_689.5577m/z	14.21652	689.5577	-2.20243	Pos	SM(d16:1/17:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C38H77N2O6P
9.79_348.2506n	9.79485	366.2844	-1.64784	Pos	Sativic acid	Lipids and lipid-like molecules	Fatty Acyls	Octadecanoids	C18H36O6
12.61_299.2575m/z	12.6132	299.2575	-1.8117	Pos	9-hydroxy-12Z-octadecenoic acid	Unclassified	Unclassified	Unclassified	C18H34O3
5.21_472.2516n	5.214017	490.2855	4.559385	Pos	3-Sulfodeoxycholic acid	Lipids and lipid-like molecules	Sphingolipids	Ceramides	C24H40O7S
12.79_619.2893m/z	12.79132	619.2893	0.689089	Neg	PI(20:4(5Z,8Z,11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoinositols	C29H49O12P
14.29_831.5743n	14.28892	832.58	-4.24363	Pos	PC(18:3(6Z,9Z,12Z)/22:4(7Z,10Z,13Z,16Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C48H82NO8P
12.83_703.5733m/z	12.8294	703.5733	-2.15157	Pos	SM(d17:1/17:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C39H79N2O6P
2.83_194.1152n	2.8349	195.1225	-1.16764	Pos	Tetraethylene glycol	Organic oxygen compounds	Organooxygen compounds	Ethers	C8H18O5
2.89_208.0942n	2.885533	209.1016	-2.11511	Pos	Dambonitol	Organic oxygen compounds	Organooxygen compounds	Alcohols and polyols	C8H16O6
14.09_794.5663m/z	14.08637	794.5663	-3.99065	Pos	PE-NMe2(18:4(6Z,9Z,12Z,15Z)/20:1(11Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C45H80NO8P
8.25_404.2061m/z	8.252417	404.2061	-1.617	Pos	3,5-Di-O-methyl-8-prenylafzelechin-4beta-ol	Lipids and lipid-like molecules	Polyketides	Flavonoids	C22H26O6
13.50_789.5641n	13.50448	790.5714	-3.93827	Pos	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/P-16:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C46H80NO7P
10.86_321.1344m/z	10.85543	321.1344	0.169323	Neg	4-Deacetylneosolaniol	Lipids and lipid-like molecules	Prenol lipids	Sesquiterpenoids	C17H24O7
14.33_769.5601m/z	14.33165	769.5601	-2.64006	Pos	PE-Cer(d14:1(4E)/25:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C41H83N2O6P
0.75_286.1887n	0.7469	287.1959	-2.04292	Pos	N-Acetyl-leucyl-leucine	Unclassified	Unclassified	Unclassified	C14H26N2O4
14.22_781.0517m/z	14.21652	781.0517	-0.28724	Pos	Emblicanin B	Lipids and lipid-like molecules	Fatty Acyls	Fatty alcohols	C34H20O22

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8.25_499.2545m/z	8.252417	499.2545	1.366837	Pos	Idebenone Metabolite(β-D-Glucopyranosiduronic acid, 4-hydroxy-3-(10-hydroxydecyl)-5,6-dimethoxy-	Unclassified	Unclassified	Unclassified	C25H40O11
10.43_414.2036n	10.43192	415.2108	-1.60683	Pos	Armillaripin	Lipids and lipid-like molecules	Prenol lipids	Sesquiterpenoids	C24H30O6
12.83_675.5426m/z	12.8294	675.5426	-1.47028	Pos	SM(d16:1/16:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C37H75N2O6P
11.76_622.3342m/z	11.76197	622.3342	-3.49912	Neg	OOV-PE	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C28H52NO9P
14.72_369.3509m/z	14.72103	369.3509	-1.71521	Pos	3-Deoxyvitamin D3	Lipids and lipid-like molecules	Sterol Lipids	Secosteroids	C27H44
4.26_284.2063m/z	4.26025	284.2063	-1.15287	Pos	Pentadecylic acid(d3)	Unclassified	Unclassified	Unclassified	C15H27D3O2
12.97_764.4839n	12.96873	782.5177	-0.05633	Pos	PI(P-16:0/14:1(9Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoinositols	C39H73O12P
5.10_474.2911m/z	5.098633	474.2911	-2.34177	Pos	N2'-Acetylgentamicin C1a	Unclassified	Unclassified	Unclassified	C21H41N5O8
10.49_452.3576m/z	10.48873	452.3576	-1.36584	Pos	Acetostearin	Unclassified	Unclassified	Unclassified	C23H46O7-2
11.54_634.3343m/z	11.54315	634.3343	-2.99282	Neg	OHHdiA-PE	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C30H54NO11P
5.88_501.3137m/z	5.87995	501.3137	-1.35196	Pos	Tuftsin	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues	C21H40N8O6
14.07_435.2736m/z	14.07218	435.2736	-1.26189	Pos	6'-Hydroxysimvastatin	Unclassified	Unclassified	Unclassified	C25H38O6
1.36_133.1050m/z	1.3554	133.105	3.403819	Pos	2-Heptanethiol	Organosulfur compounds	Thiols	Alkylthiols	C7H16S
5.53_391.2480m/z	5.529417	391.248	0.250225	Pos	15-deoxy-del ta12,14-Prostaglandin J2-2-glycerol ester	Lipids and lipid-like molecules	Fatty Acyls	Eicosanoids	C23H34O5
13.33_701.5580m/z	13.32888	701.558	-1.70492	Pos	1-tetradecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanol-amine	Unclassified	Unclassified	Unclassified	C39H74NO6P
15.29_493.3554n	15.29325	511.3892	4.461887	Pos	PE(P-20:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C25H52NO6P
6.35_809.4725m/z	6.34765	809.4725	-0.45314	Pos	PGP(16:0/18:2(9Z,12Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoglycerophosphates	C40H76O13P2
5.73_812.5197m/z	5.733283	812.5197	-0.64998	Pos	PS(O-16:0/20:2(11Z,14Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoserines	C42H80NO9P
5.36_842.4936m/z	5.359017	842.4936	-0.85714	Pos	PGP(16:0/18:3(6Z,9Z,12Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphog lycerophosphates	C40H74O13P2
5.42_434.2435n	5.422967	452.2772	0.281602	Pos	LysoPA(18:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphates	C21H39O7P

13.90_807.0669m/z	13.89975	807.0669	2.208341	pos	2-[7,8,9,12,13,14,17,18,19,25-decahydroxy-24-(hydroxymethyl)-4,22,27-trioxo-3,23,26-trioxahexacyclo[13.10.3.1^{2},^{6}.0^{5},^{10}.0^{11},^{28}.0^{16},^{21}]nonacosa-5(10),6,8,11,13,15(28),16,18,20-nonaen-29-yl]-3,4,5-trihydroxybenzoic acid	Phenylpropanoids and polyketides	Tannins	Hydrolyzable tannins	C34H24O22
5.72_766.4545n	5.718667	784.4886	-3.66664	Pos	PA(20:5(5Z,8Z,11Z,14Z,17Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphates	C45H67O8P
14.20_916.5241m/z	14.20182	916.5241	-1.34486	Pos	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C52H80NO8P
14.03_549.4153m/z	14.02888	549.4153	0.675344	Pos	Antibiotic X 14889D	Lipids and lipid-like molecules	Prenol lipids	Sesterterpenoids	C33H58O7
10.49_426.3322m/z	10.48873	426.3322	-1.08523	Pos	N-(9,12-octadecadienoyl)-glutamine	Lipids and lipid-like molecules	Fatty Acyls	Fatty amides	C23H40N2O4
14.29_796.5225m/z	14.28892	796.5225	-3.67713	Pos	PC(14:0/20:2(11Z,14Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C42H80NO8P
12.80_983.1112m/z	12.79865	983.1112	1.176265	Pos	(5-{4-[5,13-bis(3,4-dihydroxyphenyl)-6,9,17,19,21-pentahydroxy-4,12,14-trioxapentacyclo[11.7.1.0^{2},^{11}.0^{3},^{8}.0^{15},^{20}]henicosa-2(11),3(8),9,15(20),16,18-hexaen-18-yl]-3,5,7-trihydroxy-3,4-dihydro-2H-1-benzopyran-2-yl}-2-hydroxyphenyl)oxidanesulfonic acid	Phenylpropanoids and polyketides	Flavonoids	Biflavonoids and polyflavonoids	C45H36O21S
6.09_484.2797n	6.090383	485.2861	-0.81045	Pos	PG(16:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoglycerols	C22H45O9P
6.39_848.5122m/z	6.394667	848.5122	-3.74161	Pos	Concanamycin A	Unclassified	Unclassified	Unclassified	C46H75NO14

In ON vs. non-NAFLD, 73 differential metabolites were obtained, and 40 of them were categorized into the classification of lipids and lipid-like molecules (Supplementary Table 2-3). Of the 40 lipids and lipid-like molecules, 16 were glycerophospholipids. Glycerophospholipid metabolism (FDR P=0.004; pathway impact 0.339) was significantly altered (Figure 1C). PC (n=5), PE (n=6), LPE (n=1), PS (n=1), PA (n=1),and LPA (n=1) were identified in the glycerophospholipid metabolism pathway (Figure 2).Specific PCs (n=2), PE (n=1), and PS (n=1) showed associations with ON. A panel of the above four metabolites had an AUC of 0.70 in discriminating ON from non-NAFLD (Table 2).

5.4 Metabolite comparison LN vs. ON

The same analyses were performed for LN and ON subjects, where 27 differential metabolites were identified, and 12belonged to lipids and lipid-like molecules (Supplementary Table 2-4). Six of the 12 lipids and lipid-like molecules were glycerophospholipids. Pathway analysis showed that a significantly enriched pathway involved glycerophospholipid metabolism (FDR P=0.004; pathway impact 0.183, Figure 1D).In the glycerophospholipid metabolism pathway, choline and choline-related lipids includingPC(20:3(5Z,8Z,11Z)/0:0), PC(22:4(7Z,10Z,13Z,16Z)/0:0), LPC(20:3(8Z,11Z,14Z)), LPE(22:5(7Z,10Z,13Z,16Z,19Z)/0:0), LPA(18:2(9Z,12Z)/0:0) were identified (Figure 2). Choline was significantly higher, while other choline-related lipids weresignificantly lower in LN compared to ON (Figure 3).The associations of choline and specific PC, LPC, LPE, and LPA above with LN were further evaluated in LN vs. ON. Patients with higher levels

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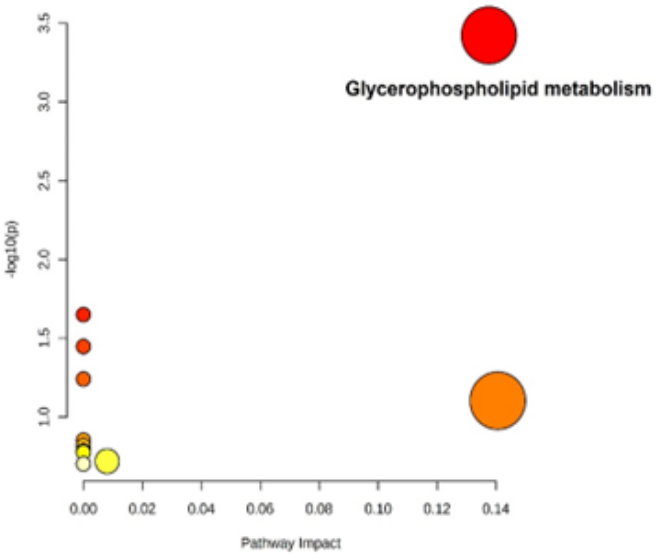
of PC and LPC had lower odds of LN. At the same time, choline was positively correlated with LN (Table 2).A panel containing choline, PC, and LPC had an AUC of 0.73 in distinguishing LN from ON. To elucidate the clinical relevance of the selected metabolites, including choline, PC, and LPC, we analyzed the relationships between these metabolites and VLDL (data not shown). A positive relationship was found between LPC(20:3(8Z,11Z,14Z)) and VLDL (OR=3.13, 95% CI:1.20-8.19).

Figure 2 (B) Lean NAFLD vs.non-NAFLD



6. Discussion

In the current cohort-based case-control pilot study conducted in China, we discovered that the glycerophospholipid metabolism pathway was altered in NAFLD vs. non-NAFLD, LN vs. non-NAFLD, and ON vs. non-NAFLD. Specific glycerophospholipids may have the potential to distinguish NAFLD and its subtypes from non-NAFLD. The glycerophospholipid metabolism pathway was also reprogrammed in LN vs. ON, and the differential metabolites included choline and specific PC, LPC, LPE, and LPA. The relative concentration of choline was obviously higher in LN, while that of the other differential metabolites was considerably lower. Choline was positively associated with LN, while specific PC and LPC were negatively associated with LN. The disruption of PC synthesis may be responsible for the development of LN. A series of metabolites and key metabolic pathways have been discovered to be influenced by NAFLD[8, 9], including phospholipid found in this study. Glycerophospholipids and PC, for example, are the most ubiquitous and abundant phospholipids in the composition of biological membranes[21-23]. The disorder of PC may lead to oxidative stress, membrane impairment, and subsequent hepatocyte injury associated with NAFLD[21-23]. As the hub of the lipid network, the metabolic reprogramming of PC can also result in the imbalance of hepatic lipid export related to NAFLD[21-23]. Although the biological function of PC is closely related to NAFLD, very few population-based studieshave reported specific PC to be associated with NAFLD[8, 9]. The study by Oresic et al. showed that a triplet of TG(16:0/18:0/18:1), PC(18:1/22:6), and PC(O-24:1/20:4) could predict NAFLD with an AUC of 0.79[24]. Our study found that a panel of six specific PCs and one PE was associated with NAFLD, with an AUC of 0.73. Similarly, diagnostic

Supplementary Table 2-2. Differential metabolites in lean NAFLD vs. non-NAFLD.

ID	Retention time (min)	m/z	Error (ppm)	Scan mode	Postulated identity	Super class	Class	Sub class	Molecule composition
12.35_549.3788n	12.35067	550.3861	-1.15616	pos	LysoPC(20:1(11Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H56NO7P
11.96_523.3631n	11.95655	546.3523	-1.37431	pos	PC(0:0/18:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H54NO7P
8.29_465.3084n	8.285067	466.3157	-1.2948	pos	Glycocholic acid	Lipids and lipid-like molecules	Steroids and steroid derivatives	Bile acids, alcohols and derivatives	C26H43NO6
11.51_478.2942m/z	11.50975	478.2942	0.643562	neg	PE(18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C23H46NO7P
13.30_551.3953n	13.2991	552.4026	0.35191	pos	LysoPC(20:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H58NO7P
11.47_521.3475n	11.46953	522.3548	-1.15072	pos	PC(18:1(11Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H52NO7P
11.26_546.3544m/z	11.26103	546.3544	-1.81846	pos	PC(20:3(5Z,8Z,11Z)/0:0)	Lipids andlipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H52NO7P
8.94_318.2997m/z	8.9353	318.2997	-2.06001	pos	17-hydroxy stearic acid	Lipids and lipid-like molecules	Fatty Acyls	Octadecanoids	C18H36O3
10.96_519.3315n	10.9624	520.3392	-1.88609	pos	PC(18:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H50NO7P

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10.85_481.3163n	10.84537	482.3235	-1.17968	pos	PC(15:0/0:0)	Lipids andlipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C23H48NO7P
11.08_507.3318n	11.07903	508.3391	-1.40844	pos	PC(17:1(10Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C25H50NO7P
11.16_569.3474n	11.15525	570.3547	-1.24228	pos	LysoPC(22:5(4Z,7Z, 10Z,13Z,16Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C30H52NO7P
11.01_612.3311m/z	11.00758	612.3311	0.642615	neg	LysoPC(22:6(4Z,7Z, 10Z,13Z,16Z,19Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C30H50NO7P
10.96_567.3317n	10.9624	568.339	-1.37532	pos	PC(22:6(4Z,7Z,10Z ,13Z,16Z,19Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C30H50NO7P
10.99_543.3318n	10.99095	544.339	-1.3519	pos	LysoPC(20:4(5Z,8Z, 11Z,14Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C28H50NO7P
11.69_547.3630n	11.69448	548.3703	-1.35738	pos	LysoPC(20:2(11Z,14Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C28H54NO7P
11.02_564.3309m/z	11.02308	564.3309	0.41043	neg	2-linoleoyl-sn-glycero -3-phosphocholine	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C26H50NO7P
11.32_590.3466m/z	11.32428	590.3466	0.553556	neg	LysoPC(20:3(8Z,11Z, 14Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C28H52NO7P
1.36_132.1018m/z	1.3554	132.1018	-0.59934	pos	L-Isoleucine	Organic acids and derivatives	Carboxylic acids and de-rivatives	Amino acids, pep tides, and analogues	C6H13NO2
10.77_400.3414m/z	10.7676	400.3414	-1.78694	pos	Palmitoylcarnitine	Benzenoids	Phenols	Benzenediols	C23H45NO4
11.04_588.3309m/z	11.03847	588.3309	0.438121	neg	LysoPC(20:4(8Z,11Z, 14Z,17Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C28H50NO7P
11.71_507.3687n	11.70948	508.3759	-0.41973	pos	PC(P-18:0/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C26H54NO6P
10.87_426.3570m/z	10.87407	426.357	-1.77611	pos	Oleoylcarnitine	Lipids and lipid-like molecules	Fatty Acyls	Fatty acid esters	C25H47NO4
11.54_566.3467m/z	11.54315	566.3467	0.687278	neg	PC(18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C26H52NO7P
11.76_554.3465m/z	11.76197	554.3465	0.382928	neg	PC(O-16:0/1:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C25H52NO7P
12.46_594.3783m/z	12.46217	594.3783	1.174066	neg	PC(20:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C28H56NO7P
11.16_540.3311m/z	11.1569	540.3311	0.8008	neg	PE(19:0/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophosphoeth anolamines	C24H50NO7P
10.91_526.3153m/z	10.9074	526.3153	0.497215	neg	PE(18:0/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophosphoeth anolamines	C23H48NO7P
11.78_592.3623m/z	11.77878	592.3623	0.535131	neg	PC(20:2(11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C28H54NO7P
11.80_552.3679m/z	11.79572	552.3679	1.543731	neg	PC(O-18:1(11Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C26H54NO6P
10.95_501.2851n	10.94828	502.2923	-0.97521	pos	PE(20:4(8Z,11Z,14Z, 17Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophosphoeth anolamines	C25H44NO7P
11.56_524.3361m/z	11.55938	524.3361	0.583331	neg	PC(P-16:0/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho cholines	C24H50NO6P
11.21_614.3469m/z	11.20773	614.3469	0.952764	neg	OHOOA-PE	Lipids and lipid-like molecules	Glycerophos-pholipids	Oxidized glycero phospholipids	C31H56NO10P

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12.95_271.2280m/z	12.94678	271.228	0.53474	neg	2-hydroxyhexadecanoic acid	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates	C16H32O3
11.87_535.3633n	11.86605	536.3706	-0.87378	pos	PC(19:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C27H54NO7P
0.69_104.1072m/z	0.691517	104.1072	2.278739	pos	Choline	Organic nitrogen compounds	Organonitrogen compounds	Quaternary ammonium salts	C5H13NO
1.15_164.0471n	1.151517	182.081	-1.26957	pos	3-(2-hydroxyphenyl) oxirane-2-carbaldehyde	Benzenoids	Phenols	1-hydroxy-4-unsubstituted benzenoids	C9H8O3
11.01_500.2787m/z	11.00758	500.2787	0.773534	neg	LysoPE(20:4(5Z,8Z,11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C25H44NO7P
12.44_507.3683n	12.44163	508.3756	-1.09294	pos	PC(O-18:1(1E)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H54NO6P
11.02_504.3103m/z	11.02308	504.3103	1.440618	neg	PC(17:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C25H48NO7P
12.69_537.3788n	12.69005	538.3861	-1.12052	pos	PC(19:0/0:0)[U]	Unclassified	Unclassified	Unclassified	C27H56NO7P
11.48_479.3369n	11.48357	480.3442	-1.36717	pos	PC(O-16:1(9E)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C24H50NO6P
11.46_438.2973m/z	11.45552	438.2973	-1.3741	pos	CPA(18:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphates	C21H41O6P
2.14_148.0523n	2.14475	166.086	-0.57959	pos	3,4-Dihydro-2H-1-benzopyran-2-one	Phenylpropanoids and polyketides	3,4-dihydrocoumarins	Unclassified	C9H8O2
10.59_424.3412m/z	10.59135	424.3412	-2.04401	pos	2-Hydroxy-3-methoxyestrone	Lipids and lipid-like molecules	Steroids and steroid derivatives	Estrane steroids	C25H47NO5
12.50_509.3842n	12.50452	510.3915	-0.66697	pos	2-O-ethyl PAF C-16	Unclassified	Unclassified	Unclassified	C26H56NO6P
12.41_466.3288m/z	12.41083	466.3288	-0.85935	pos	PE(P-18:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C23H48NO6P
11.68_464.3131m/z	11.67968	464.3131	-0.87694	pos	Dihomo-gamma-linolenoyl dopamine	Lipids and lipid-like molecules	Fatty Acyls	Fatty amides	C28H43NO3
12.22_463.2917m/z	12.22382	463.2917	1.064038	neg	Palmitoyl glucuronide	Lipids and lipid-like molecules	Fatty Acyls	Fatty acyl glycosides	C22H42O7
14.22_689.5577m/z	14.21652	689.5577	-2.20243	pos	SM(d16:1/17:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C38H77N2O6P
13.86_445.3325m/z	13.8572	445.3325	0.495309	neg	25-Hydroxytachysterol3	Lipids and lipid-like molecules	Steroids and steroid derivatives	Vitamin D and derivatives	C27H44O2
4.07_187.0631n	4.071767	205.0969	-1.3685	pos	Indoleacrylic acid	Organoheterocyclic compounds	Indoles and derivatives	Indoles	C11H9NO2
12.79_619.2893m/z	12.79132	619.2893	0.689089	neg	PI(20:4(5Z,8Z,11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoinositols	C29H49O12P
14.29_831.5743n	14.28892	832.58	-4.24363	pos	PC(18:3(6Z,9Z,12Z)/22:4(7Z,10Z,13Z,16Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C48H82NO8P
12.74_595.2894m/z	12.73935	595.2894	0.843377	neg	PI(18:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoinositols	C27H49O12P

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12.53_464.3148m/z	12.53142	464.3148	0.328307	neg	PE(O-18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C23H48NO6P
11.47_550.3148m/z	11.46953	550.3148	1.500814	pos	PG-PE	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C26H50NO10P
12.48_489.3073m/z	12.4795	489.3073	0.904967	neg	2-Stearyl citrate	Organic acids and derivatives	Carboxylic acids and derivatives	Tricarboxylic acids and derivatives	C24H44O7
11.02_632.3185m/z	11.02308	632.3185	-3.17129	neg	PHOOA-PS	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C30H54NO12P
11.88_511.2916m/z	11.88175	511.2916	0.652895	neg	(24R)-11alpha,20,24-trihydroxyecdysone	Lipids and lipid-like molecules	Sterol Lipids	Sterols	C27H44O9
14.47_381.1739m/z	14.46988	381.1739	-0.55846	neg	S-Japonin	Lipids and lipid-like molecules	Prenol lipids	Sesquiterpenoids	C19H28O3S
12.83_675 5426m/z	12.8294	675.5426	-1.47028	pos	SM(d16:1/16:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C37H75N2O6P
11.54_634.3343m/z	11.54315	634.3343	-2.99282	neg	OHHdiA-PE	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C30H54NO11P
10.91_632.3187m/z	10.9074	632.3187	-2.80317	neg	OKHdiA-PE	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C30H52NO11P
11.54_583.3368m/z	11.54315	583.3368	-0.43049	neg	Thioperamide	Unclassified	Unclassified	Unclassified	C15H24N4S
10.98_548.2981m/z	10.97652	548.2981	-0.42863	pos	PS(20:3(8Z,11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoserines	C26H46NO9P
14.27_355.1583m/z	14.267	355.1583	0.051248	neg	Hydroxyzine	Benzenoids	Benzene and substituted derivatives	Diphenylmethanes	C21H27ClN2O2
1.08_137.0456m/z	1.082017	137.0456	2.294357	pos	1-Pentanesulfenothioic acid	Organosulfur compounds	Sulfenyl compounds	Unclassified	C5H12S2
7.64_514.2844m/z	7.643067	514.2844	-0.04979	neg	Tauro-b-muricholic acid	Lipids and lipid-like molecules	Steroids and steroid derivatives	Bile acids, alcohols and derivatives	C26H45NO7S
14.20_916.5241m/z	14.20182	916.5241	-1.34486	pos	PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C52H80NO8P

panels containing specific glycerophospholipids at the center of PC were revealed for both LN and ON, further implicating the importance of PC and its related metabolites along the pathway in the pathogenesis of NAFLD and the diagnostic potential of specific glycerophospholipids.

Apart fromthe overall NAFLD, the metabolism of glycerophospholipid was also reprogrammed when comparing two NAFLD subtypes, LN vs. ON. The differential metabolites included choline and specific PC, LPC, LPE, and LPA. LN had significantly increased choline and decreased specific PC, LPC, LPE, and LPA than ON, indicating that the production of PC from various routes starting from choline, PE, and PA was reduced (SupplementaryFigure 3). Accordingly, we observed that choline was positively associated with LN while PC and its derivative LPC were negatively associated with LN, suggesting that choline, PC, and LPC might be a hallmark of LN. Choline is an essential nutrient and may synthesize PC through multiple routes[21-23].The significantly higher relative abundance of choline but lower PC in LN than ON may be due to decreased bioavailability of serum choline in LN[25]. Decreased PC may further result in NAFLD through membrane instability, oxidative stress, endoplasmic reticulum stress, ordecreased VLDL assembly and export[21-23]. An experimental study[26] showed that betaine homocysteine methyltransferase gene knockout mice and PE N-methyltransferase gene knockout mice had decreased liver choline, increased energy consumption, and less weight gain compared with wild-type mice. In other words,decreased bioavailability of serum choline in LN may subsequently increase energy consumption and contribute to obesity resistance in patients with LN. Population-based genetic findings also implicated the indispensable role of PC-synthesizing enzyme in the occurrence of LN [27]. The aforementioned hypothesis is also supported

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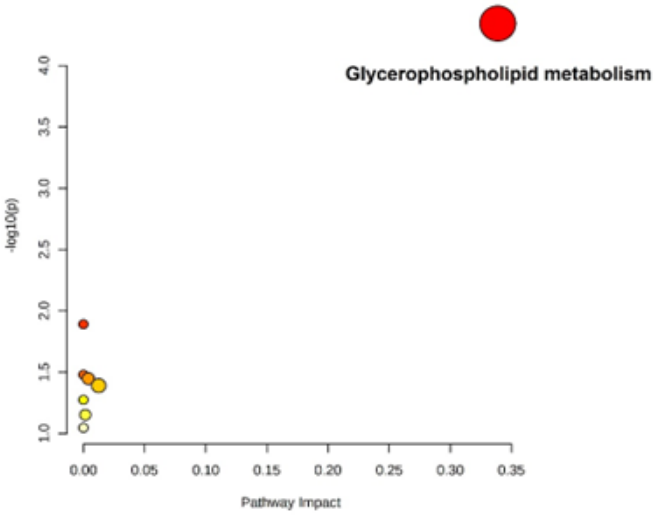
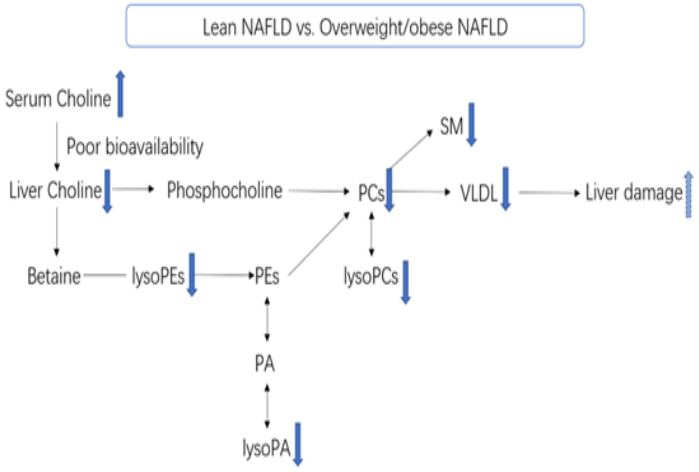
by our results, i.e., decreased LPC is related to decreased VLDL secretion and increase the odds of developing LN, and a decreased trend of VLDL in LN compared to ON was found though not statistically significant. Based on the existing evidence, we considered that choline and choline-related metabolites along the pathway of PC production might mediate the pathogenesis of LN.

Figure 3 (C) Overweight/obese NAFLD vs.non-NAFLD



We observed that LN subjects featured higher serum choline, lower specific PC, lower blood lipids such as TG, VLDL, and lower insulin resistance indicators than ON. Alonso et al.[28] discovered two NAFLD subtypes in humans: The m-subtype of NAFLD showed decreased PC synthesis and impaired VLDL secretion, which was similar to the LN phenotype in our study, while the non-M-subtype had a phenotype of increased liver lipid de novo synthesis, normal VLDL secretion, and increased serum TG and TC levels, in line with ON phenotype. Also, the feature of LN in our study seemed to be consistent with the genetic subtype of NAFLD [29], which is characterized by impaired hepatic mitochondrial function, no insulin resistance and decreased VLDL secretion. This could be supported by the fact that LN subjects may have a higher frequency of PNPLA3 rs738409 GG genotype [30, 31] and TM6SF2 rs58542926 T allele [16, 32] than ON. In contrast, the metabolic component of NAFLD [29], corresponding to ON, is characterized by insulin resistance, hepatic oversupply of substrates, and increased VLDL secretion. Therefore, these findings may indicate that LN is generated by decreased PC synthesis and hepatic VLDL secretion or impaired hepatic mitochondrial function, whereas liver lipid de novo synthesis or insulin resistance may play a vital part in the onset of ON. We need further study to determine whether VLDL secretion is reduced in LN and, in contrast, hepatic lipid de novo synthesis is upregulated in ON [Supplementary Figure 3]. Our study still has several limitations. First, the current sample size is relatively small because of the pilot study's exploratory nature, which may lead tothe wide CIsof selected metabolite, but still goodmodeldiscriminationis achieved. Second, untargeted metabolomics was used to explore the candidate metabolites with only relative abundance obtained.



Supplementary Figure 3. Description of the glycerophospholipid metabolism pathway-related metabolites changes in lean NAFLD vs. overweight/obese NAFLD.

Lean NAFLD had significantly increased choline and decreased specific PC, LPC, LPE, and LPA than overweight/obese NAFLD. The significantly higher concentration of choline but lower PC in lean NAFLD than in overweight/obese NAFLD may be due to the decreased bioavailability of serum choline in lean NAFLD. Decreased PC may further result in decreased VLDL and the development of liver damage.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; PC, phosphatidylcholine; LysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LysoPE, lysophosphatidylethanolamine; PA, phosphatidic acid; LPA, lysophosphatidic acid; VLDL, very-low-density lipoprotein.

Further targeted metabolomics detection is needed to accurately quantify the candidate metabolites for diagnostic applications and for the study reproducibilityin general [8]. Third, the inaccuracy of ultrasound in fatty liver diagnosis may result in non-differential misclassification and underestimation of the actual effect size.Despite possible sample overlap and model overfitting, significantly differential metabolites do exist between groups.Last but not least, the underlying mechanisms of the candidate metabolites driving NAFLD, LN, and ON awaits further investigation. We assume that under the obesogenic stress, compared with ON patients, LN patients may have different metabolic adaptations probably arising from intra-individual variations in gene or microbiome[33], which leads to the reprogramming of choline and glycerophospholipid metabolism, and this metabolic adaptation may possibly influence some unknown factors for example, membrane stability or protein[34] and finally results in the characteristics and long-term outcomes of LN(Supplementary Figure 4).

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Supplementary Table 2-3. Differential metabolites in overweight/obese NAFLD vs. non-NAFLD.

ID	Retention time (min)	m/z	Error (ppm)	Scan mode	Postulated identity	Super class	Class	Sub class	Molecule composition
12.35_549 .3788n	12.35067	550.3861	-1.15616	pos	LysoPC(20:1(11Z))	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C28H56NO7P
11.96_523 .3631n	11.95655	546.3523	-1.37431	pos	PC(0:0/18:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C26H54NO7P
8.29_465 .3084n	8.285067	466.3157	-1.2948	pos	Glycocholic acid	Lipids and lipid-like molecules	Steroids and steroid deriva tives	Bile acids, alco hols and deriva tives	C26H43NO6
11.51_478 .2942m/z	11.50975	478.2942	0.643562	neg	PE(18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycero phosphoethanol amines	C23H46NO7P
13.30_551 .3953n	13.2991	552.4026	0.35191	pos	LysoPC(20:0/0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C28H58NO7P
11.47_521 .3475n	11.46953	522.3548	-1.15072	pos	PC(18:1(11Z)/0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C26H52NO7P
11.26_546 .3544m/z	11.26103	546.3544	-1.81846	pos	PC(20:3(5Z,8Z,11Z) /0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C28H52NO7P
8.94_318 .2997m/z	8.9353	318.2997	-2.06001	pos	17-hydroxy stearic acid	Lipids and lipid-like molecules	Fatty Acyls	Octadecanoids	C18H36O3
10.96_519 .3315n	10.9624	520.3392	-1.88609	pos	PC(18:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C26H50NO7P
10.85_481 .3163n	10.84537	482.3235	-1.17968	pos	PC(15:0/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C23H48NO7P
11.08_507 .3318n	11.07903	508.3391	-1.40844	pos	PC(17:1(10Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C25H50NO7P
11.16_569 .3474n	11.15525	570.3547	-1.24228	pos	LysoPC(22:5(4Z,7Z, 10Z,13Z,16Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C30H52NO7P
11.01_612 .3311m/z	11.00758	612.3311	0.642615	neg	LysoPC(22:6(4Z,7Z, 10Z13Z,16Z,19Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C30H50NO7P
10.96_567 .3317n	10.9624	568.339	-1.37532	pos	PC(22:6(4Z,7Z,10Z, 13Z,16Z,19Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C30H50NO7P
10.99_543 .3318n	10.99095	544.339	-1.3519	pos	LysoPC(20:4(5Z,8Z, 11Z,14Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophospho-cholines	C28H50NO7P
11.69_547 .3630n	11.69448	548.3703	-1.35738	pos	LysoPC(20:2(11Z ,14Z))	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho-cholines	C28H54NO7P
11.02_564 .3309m/z	11.02308	564.3309	0.41043	neg	2-linoleoyl-sn-glycero-3-phosphocholine	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho-cholines	C26H50NO7P
11.32_590 .3466m/z	11.32428	590.3466	0.553556	neg	LysoPC(20:3(8Z,11Z ,14Z))	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho-cholines	C28H52NO7P
1.36_132 .1018m/z	1.3554	132.1018	-0.59934	pos	L-Isoleucine	Organic acids and de-rivatives	Carboxylic ac ids and deriva tives	Amino acids, peptides, and an-alogues	C6H13NO2
10.77_400 .3414m/z	10.7676	400.3414	-1.78694	pos	Palmitoylcarnitine	Benzenoids	Phenols	Benzenediols	C23H45NO4
11.04_588 .3309m/z	11.03847	588.3309	0.438121	neg	LysoPC(20:4(8Z,11Z, 14Z,17Z))	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C28H50NO7P

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11.71_507.3687n	11.70948	508.3759	-0.41973	pos	PC(P-18:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H54NO6P
10.87_426.3570m/z	10.87407	426.357	-1.77611	pos	Oleoylcarnitine	Lipids and lipid-like molecules	Fatty Acyls	Fatty acid esters	C25H47NO4
11.54_566.3467m/z	11.54315	566.3467	0.687278	neg	PC(18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H52NO7P
11.76_554.3465m/z	11.76197	554.3465	0.382928	neg	PC(O-16:0/1:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C25H52NO7P
12.46_594.3783m/z	12.46217	594.3783	1.174066	neg	PC(20:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H56NO7P
11.16_540.3311m/z	11.1569	540.3311	0.8008	neg	PE(19:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C24H50NO7P
10.91_526.3153m/z	10.9074	526.3153	0.497215	neg	PE(18:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C23H48NO7P
11.78_592.3623m/z	11.77878	592.3623	0.535131	neg	PC(20:2(11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C28H54NO7P
11.80_552.3679m/z	11.79572	552.3679	1.543731	neg	PC(O-18:1(11Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H54NO6P
10.95_501.2851n	10.94828	502.2923	-0.97521	pos	PE(20:4(8Z,11Z,14Z,17Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C25H44NO7P
11.56_524.3361m/z	11.55938	524.3361	0.583331	neg	PC(P-16:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C24H50NO6P
11.21_614.3469m/z	11.20773	614.3469	0.952764	neg	OHOOA-PE	Lipids and lipid-like molecules	Glycerophospholipids	Oxidized glycerophospholipids	C31H56NO10P
12.95_271.2280m/z	12.94678	271.228	0.53474	neg	2-hydroxyhexadecanoic acid	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates	C16H32O3
11.87_535.3633n	11.86605	536.3706	-0.87378	pos	PC(19:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C27H54NO7P
0.69_104.1072m/z	0.691517	104.1072	2.278739	pos	Choline	Organic nitrogen compounds	Organonitrogen compounds	Quaternary ammonium salts	C5H13NO
1.15_164.0471n	1.151517	182.081	-1.26957	pos	3-(2-hydroxyphenyl) oxirane-2-carbaldehyde	Benzenoids	Phenols	1-hydroxy-4-unsubstituted benzenoids	C9H8O3
11.01_500.2787m/z	11.00758	500.2787	0.773534	neg	LysoPE(20:4(5Z,8Z,11Z,14Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoethanolamines	C25H44NO7P
12.44_507.3683n	12.44163	508.3756	-1.09294	pos	PC(O-18:1(1E)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C26H54NO6P
11.02_504.3103m/z	11.02308	504.3103	1.440618	neg	PC(17:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C25H48NO7P
12.69_537.3788n	12.69005	538.3861	-1.12052	pos	PC(19:0/0:0)[U]	Unclassified	Unclassified	Unclassified	C27H56NO7P
11.48_479.3369n	11.48357	480.3442	-1.36717	pos	PC(O-16:1(9E)/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C24H50NO6P
11.46_438.2973m/z	11.45552	438.2973	-1.3741	pos	CPA(18:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphates	C21H41O6P

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2.14_148 .0523n	2.14475	166.086	-0.57959	pos	3,4-Dihydro-2H-1 -benzopyran-2-one	Phenylpropanoids and polyketides	3,4-dihydro-coumarins	Unclassified	C9H8O2
10.59_424 .3412m/z	10.59135	424.3412	-2.04401	pos	2-Hydroxy-3-methoxyestrone	Lipids and lipid-like molecules	Steroids and steroid derivatives	Estrane steroids	C25H47NO5
12.50_509 .3842n	12.50452	510.3915	-0.66697	pos	2-O-ethyl PAF C-16	Unclassified	Unclassified	Unclassified	C26H56NO6P
12.41_466 .3288m/z	12.41083	466.3288	-0.85935	pos	PE(P-18:0/0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycero phospho ethanolamines	C23H48NO6P
11.68_464 .3131m/z	11.67968	464.3131	-0.87694	pos	Dihomo-gamma-linolenoyl dopamine	Lipids and lipid-like molecules	Fatty Acyls	Fatty amides	C28H43NO3
12.22_463 .2917m/z	12.22382	463.2917	1.064038	neg	Palmitoyl glucuronide	Lipids and lipid-like molecules	Fatty Acyls	Fatty acyl glycosides	C22H42O7
14.22_689 .5577m/z	14.21652	689.5577	-2.20243	pos	SM(d16:1/17:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C38H 77N2O6P
13.86_445 .3325m/z	13.8572	445.3325	0.495309	neg	25-Hydroxytachysterol3	Lipids and lipid-like molecules	Steroids and ste roid derivatives	Vitamin D and derivatives	C27H44O2
4.07_187 .0631n	4.071767	205.0969	-1.3685	pos	Indoleacrylic acid	Organoheterocyclic compounds	Indoles and derivatives	Indoles	C11H9NO2
12.79_619 .2893m/z	12.79132	619.2893	0.689089	neg	PI(20:4(5Z,8Z,11Z,14Z) /0:0)	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphoinositols	C29H49O12P
14.29_831 .5743n	14.28892	832.58	-4.24363	pos	PC(18:3(6Z,9Z,12Z)/22 :4(7Z,10Z,13Z,16Z))	Lipids and lipid-like molecules	Glycerophospholipids	Glycerophosphocholines	C48H82NO8P
12.74_595 .2894m/z	12.73935	595.2894	0.843377	neg	PI(18:2(9Z,12Z)/0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho inositols	C27H49O12P
12.53_464 .3148m/z	12.53142	464.3148	0.328307	neg	PE(O-18:1(9Z)/0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycero phosphoethanol amines	C23H48NO6P
11.47_550 .3148m/z	11.46953	550.3148	1.500814	pos	PG-PE	Lipids and lipid-like molecules	Glycerophos pholipids	Oxidized glycer ophospholipids	C26H 50NO10P
12.48_489 .3073m/z	12.4795	489.3073	0.904967	neg	2-Stearyl citrate	Organic acids and derivatives	Carboxylic acids and deriv atives	Tricarboxylic acids and deriva tives	C24H44O7
11.02_632 .3185m/z	11.02308	632.3185	-3.17129	neg	PHOOA-PS	Lipids and lipid-like molecules	Glycerophos pholipids	Oxidized glycer ophospholipids	C30H 54NO12P
11.88_511 .2916m/z	11.88175	511.2916	0.652895	neg	(24R)-11alpha,20,24 -trihydroxyecdysone	Lipids and lipid-like molecules	Sterol Lipids	Sterols	C27H44O9
14.47_381 .1739m/z	14.46988	381.1739	-0.55846	neg	S-Japonin	Lipids and lipid-like molecules	Prenol lipids	Sesquiterpenoids	C19H28O3S
12.83_675 .5426m/z	12.8294	675.5426	-1.47028	pos	SM(d16:1/16:0)	Lipids and lipid-like molecules	Sphingolipids	Phosphosphingolipids	C37H 75N2O6P
11.54_634 .3343m/z	11.54315	634.3343	-2.99282	neg	OHHdiA-PE	Lipids and lipid-like molecules	Glycerophos pholipids	Oxidized glycerophospholipids	C30H 54NO11P
10.91_632 .3187m/z	10.9074	632.3187	-2.80317	neg	OKHdiA-PE	Lipids and lipid-like molecules	Glycerophos pholipids	Oxidized glycer ophospholipids	C30H 52NO11P
11.54_583 .3368m/z	11.54315	583.3368	-0.43049	neg	Thioperamide	Unclassified	Unclassified	Unclassified	C15H24N4S
10.98_548 .2981m/z	10.97652	548.2981	-0.42863	pos	PS(20:3(8Z,11Z,14Z) /0:0)	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophos phoserines	C26H46NO9P

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14.27_355 .1583m/z	14.267	355.1583	0.051248	neg	Hydroxyzine	Benzenoids	Benzene and substituted derivatives	Diphenylmeth anes	C21H27Cl N2O2
1.08_137 .0456m/z	1.082017	137.0456	2.294357	pos	1-Pentanesulfenothioic acid	Organosulfur com-pounds	Sulfenyl compounds	Unclassified	C5H12S2
7.64_514 .2844m/z	7.643067	514.2844	-0.04979	neg	Tauro-b-muricholic acid	Lipids and lipid-like molecules	Steroids and steroid deriva tives	Bile acids, alco hols and deriva tives	C26H 45NO7S
14.20_916 .5241m/z	14.20182	916.5241	-1.34486	pos	PC(22:6(4Z,7Z,10Z,13Z ,16Z,19Z)/22:6(4Z,7Z, 10Z,13Z,16Z,19Z))	Lipids and lipid-like molecules	Glycerophos pholipids	Glycerophospho cholines	C52H80NO8P

Figure 4 (D) Lean NAFLD vs.Overweight/obese NAFLD

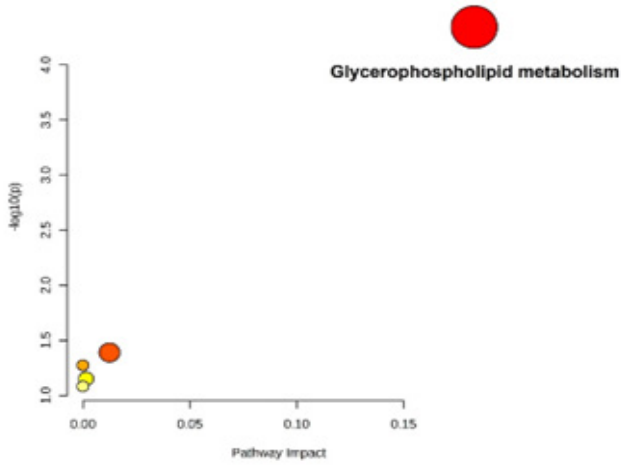

Figure 1. Pathway analyses of thedifferential metabolitesin various comparisons.

The bubble plot indicated alterations in the primary metabolic pathways,with impact and P-value attributed to the accumulation of differential metabolites along each enriched pathway.
The bubble

In conclusion,alterations in the glycerophospholipid metabolism pathway may be principal in NAFLD and its subtypes.Specific glycerophospholipids may have the potential to distinguish NAFLD and its subtypes from non-NAFLD. The disruption of PC synthesis may be responsible for the development of LN.Our results have translational implications in dietary recommendations and interventions for lean NAFLD patients, who would probably benefit from more PC intake and supplementation with diet. Further targeted metabolomics studies with larger sample size and in-vitro functional validation are needed to investigate the effect and mechanisms of choline and choline-related metabolites on NAFLD and its subtypes, especially on LN, and develop potential clinically relevant targets for early intervention and treatment.

Supplementary Table 2-4. Differential metabolites in lean NAFLD vs. overweight/obese NAFLD.

ID	Retention time (min)	m/z	Error (ppm)	Scan mode	Postulated identity	Super class	Class	Sub class	Molecule com-position
11.26_546 .3544m/z	11.26103	546.354	-1.81846	pos	PC(20:3(5Z,8Z,11Z) /0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophos-phocholines	C28H52NO7P
11.32_527 .3006n	11.31937	528.308	-1.10199	pos	LysoPE(22:5(7Z,10Z, 13Z,16Z,19Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycero phosphoetha nolamines	C27H46NO7P
11.32_590 .3466m/z	11.32428	590.347	0.553556	neg	LysoPC(20:3(8Z,11Z ,14Z))	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophos-phocholines	C28H52NO7P
11.64_616 .3625m/z	11.64277	616.363	0.945009	neg	PC(22:4(7Z,10Z,13Z ,16Z)/0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophos-phocholines	C30H54NO7P
13.46_269 .2259m/z	13.46043	269.226	-1.77755	pos	Anhydroretinol	Lipids and lipid-like molecules	Prenol lipids	Sesquiter penoids	C20H28

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8.25_390 .1905m/z	8.252417	390.19	-1.75623	pos	1-hydroxy-2-[6-(2-methylbut-3-en-2-yl) -7-oxo-2H,3H,7H-furo [3,2-g]chromen-2-yl] propan-2-yl acetate	Phenylpropanoids and polyketides	Coumarinsand derivatives	Furanocouma-rins	C21H24O6
4.77_326 .1935n	4.772317	344.227	-1.70124	pos	Heptaethylene glycol	Organic oxygen compounds	Organoox ygen compounds	Ethers	C14H30O8
4.93_370 .2197n	4.9328	388.254	-1.61355	pos	Octaethylene glycol	Organic oxygen compounds	Organooxygen compounds	Ethers	C16H34O9
4.58_282 .1673n	4.576767	300.201	-1.89426	pos	Hexaethylene glycol	Organic oxygen compounds	Organooxygen compounds	Ethers	C12H26O7
14.25_433 .2595m/z	14.25007	433.26	-0.07154	neg	3alpha-Hydroxy-12-oxo-5 beta-chol-7-en-24-oic Acid	Unclassified	Unclassified	Unclassified	C24H36O4
11.68_320 .2353n	11.6774	319.228	0.341611	neg	(±)12-HETE	Unclassified	Unclassified	Unclassified	C20H32O3
11.60_280 .2398n	11.6025	303.231	-1.64985	pos	Mangiferic acid	Lipids and lipid-like molecules	Fatty Acyls	Lineolic acids and derivatives	C18H32O2
0.69_104 .1072m/z	0.691517	104.107	2.278739	pos	Choline	Organic nitrogen compounds	Organonitrogen compounds	Quaternary am-monium salts	C5H13NO
4.83_212 .0017m/z	4.82885	212.002	-2.90094	neg	Indoxyl sulfate	Organic acids and derivatives	Organic sulfuric acids and deriv-atives	Arylsulfates	C8H7NO4S
8.25_358 .1410n	8.252417	376.175	-1.69737	pos	4,8,11-trihydroxy-17 -methoxy-2-oxatricy clo[13.2.2.1^{3},^{7}]icosa-1(17),3,5,7(20),15,18-hexaen-10-one	Phenylpropanoids and polyketides	Diarylhepta noids	Cyclic diaryl heptanoids	C20H22O6
2.34_99 .0687n	2.341183	100.076	3.058994	pos	δ-Valerolactam	Organoheterocyclic compounds	Piperidines	Piperidinones	C5H9NO
4.34_312 .1647m/z	4.341433	312.165	-2.1445	pos	Ethyl (S)-3-hydroxy butyrate glucoside	Lipids and lipid-like molecules	Fatty Acyls	Fatty acyl glycosides	C12H22O8
13.86_445 .3325m/z	13.8572	445.333	0.495309	neg	25-Hydroxytachysterol3	Lipids and lipid-like molecules	Steroids and ste-roid derivatives	Vitamin D and derivatives	C27H44O2
2.89_208 .0942n	2.885533	209.102	-2.11511	pos	Dambonitol	Organic oxygen compounds	Organooxygen compounds	Alcohols and polyols	C8H16O6
8.25_404 .2061m/z	8.252417	404.206	-1.617	pos	3,5-Di-O-methyl-8-pre nylafzelechin-4beta-ol	Lipids and lipid-like molecules	Polyketides	Flavonoids	C22H26O6
10.86_321 .1344m/z	10.85543	321.134	0.169323	neg	4-Deacetylneosolaniol	Lipids and lipid-like molecules	Prenol lipids	Sesquiter penoids	C17H24O7
8.25_499 .2545m/z	8.252417	499.254	1.366837	pos	Idebenone Metabolite (β-D-Glucopyranosiduronic acid, 4-hydroxy-3-(10-hy droxydecyl)-5,6-dimethoxy-	Unclassified	Unclassified	Unclassified	C25H40O11
13.87_813 .6814m/z	13.87095	813.681	-3.6433	pos	SM(d18:1/24:1(15Z))	Lipids and lipid-like molecules	Sphingolipids	Phosphosphin-golipids	C47H 93N2O6P
4.26_284 .2063m/z	4.26025	284.206	-1.15287	pos	Pentadecylic acid(d3)	Unclassified	Unclassified	Unclassified	C15H27D3O2
5.10_474 .2911m/z	5.098633	474.291	-2.34177	pos	N2'-Acetylgentamicin C1a	Unclassified	Unclassified	Unclassified	C21H41N5O8

14.07_435 .2736m/z	14.07218	435.274	-1.26189	pos	6'-Hydroxysimvastatin	Unclassified	Unclassified	Unclassified	C25H38O6
5.42_434 .2435n	5.422967	452.277	0.281602	pos	LysoPA(18:2(9Z,12Z) /0:0)	Lipids and lipid-like molecules	Glycerophos-pholipids	Glycerophos-phates	C21H39O7P

7. Ethics approval and consent to participate

We obtained approval from the Ethics Committee of the Kailuan General Hospital and the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. In addition, we got written informed consent from all participants. Availability of data and materials The datasets used during the current study are available from the corresponding author on reasonable request. Competing interests The authors declare that they have no competing interests.

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Authors' contributions

LW and SLW designed the study. SHC collected the data. SQH processed the data. YQL and YL performed the statistical analysis. SQH and YHW interpreted the data. YQL and YL drafted the manuscript. LW and SLW revised the manuscript critically. All authors read and approved the final manuscript.

9. Abbreviations:

NAFLD, non-alcoholic fatty liver disease; BMI,body mass index; LN, lean NAFLD; ON, overweight or obese NAFLD; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; VLDL, very-low-density lipoprotein;TyG Index, triglyceride glucose index; TG/HDL-C ratio, triglyceride to high-density lipoprotein cholesterol ratio; LC-MS, liquid chromatography-tandem mass spectrometry; QC, quality control; OPLS-DA, orthogonal partial least squares discriminant analysis; VIP, variable importance in projection; OR, odds ratio; 95%CI, 95% confidence interval; AUC, area under the curve; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; LPA, lysophosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol.

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