BMC Gastroenterology

RESEARCH

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Early elevation of complement-related proteins in metabolic dysfunction-associated steatotic liver disease (MASLD) with normal liver enzymes

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Abstract

Background Metabolic dysfunction-associated steatotic liver disease (MASLD) affects a quarter of the population. Although multiple studies have investigated the mechanisms underlying steatohepatitis and MASLD progression, little attention has been paid to milder cases, particularly those with normal enzyme levels. Our objective was to evaluate the molecular events in patients with MASLD and normal enzyme levels.

Methods We performed a proteomic analysis on serum samples from 83 healthy controls and 50 patients with MASLD. The major findings were then validated in serum samples from 35 controls and 60 patients with MASLD and normal liver enzyme levels (MASLD-NLE), as well as a male mouse model with metabolic dysfunction-associated liver steatosis and normal liver enzymes (mice-NLE) induced by a high-fat diet (HFD).

Results Despite having normal enzyme levels, patients with MASLD-NLE exhibited significantly higher levels of ALT, AST, γGGT, hs-CRP, uric acid and creatine compared to controls. Serum proteomics indicated elevated complement-related proteins in patients with MASLD-NLE, evidenced by elevated protein levels of C3 and CFH in both cases using different cutoffs for normal ALT levels. C3 and CFH also showed positive correlations with γGGT, hs-CRP, ALT, and uric acid levels. Additionally, the elevation of C3 and CFH was confirmed in another set of human serum samples, as well as in serum and liver samples of mice-NLE using ELISA.

Conclusions Our study provides novel evidence that the complement system is already activated in patients with MASLD-NLE, potentially offering new avenues for treating MASLD at an early stage.

Keywords MASLD, Liver, Normal enzyme level, Complement system

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Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as nonalcoholic fatty liver disease, is the most common chronic liver disease worldwide, affecting approximately one-third of the adult population [1, 2]. In 2023, major liver associations endorsed MASLD, substituting 'steatotic' for 'fatty' to improve scientific precision and reduce stigma [3]. MASLD reverts to mutually exclusive subcategories (e.g., MASLD-alcohol, MASLD-viral), restoring etiology-specific subclassification [4]. If left untreated, MASLD can progress from simple steatosis to metabolic dysfunction-associated steatohepatitis (MASH), fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC). A nationwide cohort study based on biopsy-confirmed patients with MASLD showed an increased overall mortality rate in patients with MASLD compared to controls (28.6 versus 16.9/1000 person-year) caused by extra-hepatic cancers, cirrhosis, cardiovascular diseases, and HCC [5]. Consequently, timely treatment and disease control are crucial for improving the prognosis of patients with MASLD. However, despite the urgent need, no drugs have received approval from the Food and Drug Administration for MASLD treatment, largely due to limited understanding of the underlying pathophysiology. Therefore, it is imperative to elucidate the biological mechanisms involved in the onset and progression of MASLD.

Numerous studies have investigated the biological processes linked to the progression of MASLD. It has been observed that insulin resistance in various tissues, resulting from an imbalance in energy intake and expenditure, leads to fat accumulation in the liver. This, combined with intracellular damage and hepatic insulin resistance, potentiates further liver inflammation, fibrosis, and carcinogenesis [6]. Additionally, mitochondrial dysfunction, including impaired mitochondrial fatty acid oxidation and reduced mitochondrial quality, has been suggested to play a significant role in the development and progression of MASLD [7]. Furthermore, injured hepatocytes secrete extracellular vehicles (EVs). EVs can induce the migration of bone marrow-derived monocytes and activate liver macrophages. These EVs carry bioactive cargoes such as nucleic acids, proteins, and lipids packed in membrane-bound particles. EVs secreted by other cells can also regulate the activation of hepatic stellate cells that give rise to liver fibrosis [8]. An analysis of liver biopsies from NASH patients using single-cell transcriptomics revealed an upregulation of macrophage subpopulations and abnormalities in the complement system [9]. Aside from the focus on the host, several studies have indicated that gut microbial dysbiosis contributes to the pathogenesis of MASLD through mechanisms such as increased mucosal permeability and bacterial translocation [10]. Small intestinal bacterial overgrowth has also been reported to induce hepatic injury [11]. Moreover, gut-derived metabolites have been found to promote liver inflammation [12], and microbial-derived exosomes from the gut have been proposed to play potential roles in MASLD progression [13]. Overall, various dysfunctions have been associated with the progression of MASLD. However, limited attention has been paid to less severe cases of MASLD, particularly those before the onset of progression. Understanding the events preceding MASLD progression can lay the foundation for developing early-stage disease control protocols. Therefore, there is an urgent need to evaluate the events associated with less severe cases of MASLD.

Proteomics, which can detect large numbers and multiple types of proteins in a single run, has been successfully applied to explore the possible mechanisms of liver diseases and metabolic syndromes [14]. Montgomery et al. described the overall changes in hepatokine secretion in NASH and identified arylsulfatase A as a regulator of liver-to-muscle communication through in-depth proteomic profiling [15]. Mocciaro et al. suggested that metabolic syndrome is associated with impaired phospholipid metabolism in high-density lipoprotein cholesterol using untargeted whole-serum lipidomics, targeted proteomics, and lipoprotein lipidomics [16]. Sehgal et al. revealed that the malfunctioning of neutrophils in decompensated cirrhosis patients with sepsis could be attributed to autophagy proteins, such as DnaJ homolog subfamily C member 13 (DNAJC13) and alpha 2-HS glycoprotein (AHSG), based on plasma proteomic analysis. These proteins may serve as potential therapeutic targets [17]. Therefore, proteomics is an ideal approach for investigating the underlying mechanisms of MASLD.

In this study, we conducted a proteomic analysis to evaluate the molecular events in patients with MASLD and normal enzyme levels using serum samples obtained from healthy controls and patients with MASLD. The major findings were then validated in another set of serum samples from 35 controls and 60 patients with MASLD-NLE, as well as mice-NLE induced by a high-fat diet (HFD).

Materials and methods

Study design

Figure 1a presented the schema of the study design. This study involved proteomic analysis of serum samples obtained from a discovery set, including 83 healthy controls, 38 MASLD patients with normal liver enzyme levels (MASLD-NLE), and 12 patients with increased liver enzyme levels (MASLD-ILE). Initially, the focus was on evaluating the proteins that were perturbed in patients with MASLD-NLE compared to controls. The dynamics of these differentially expressed proteins during MASLD progression were also investigated using serum samples

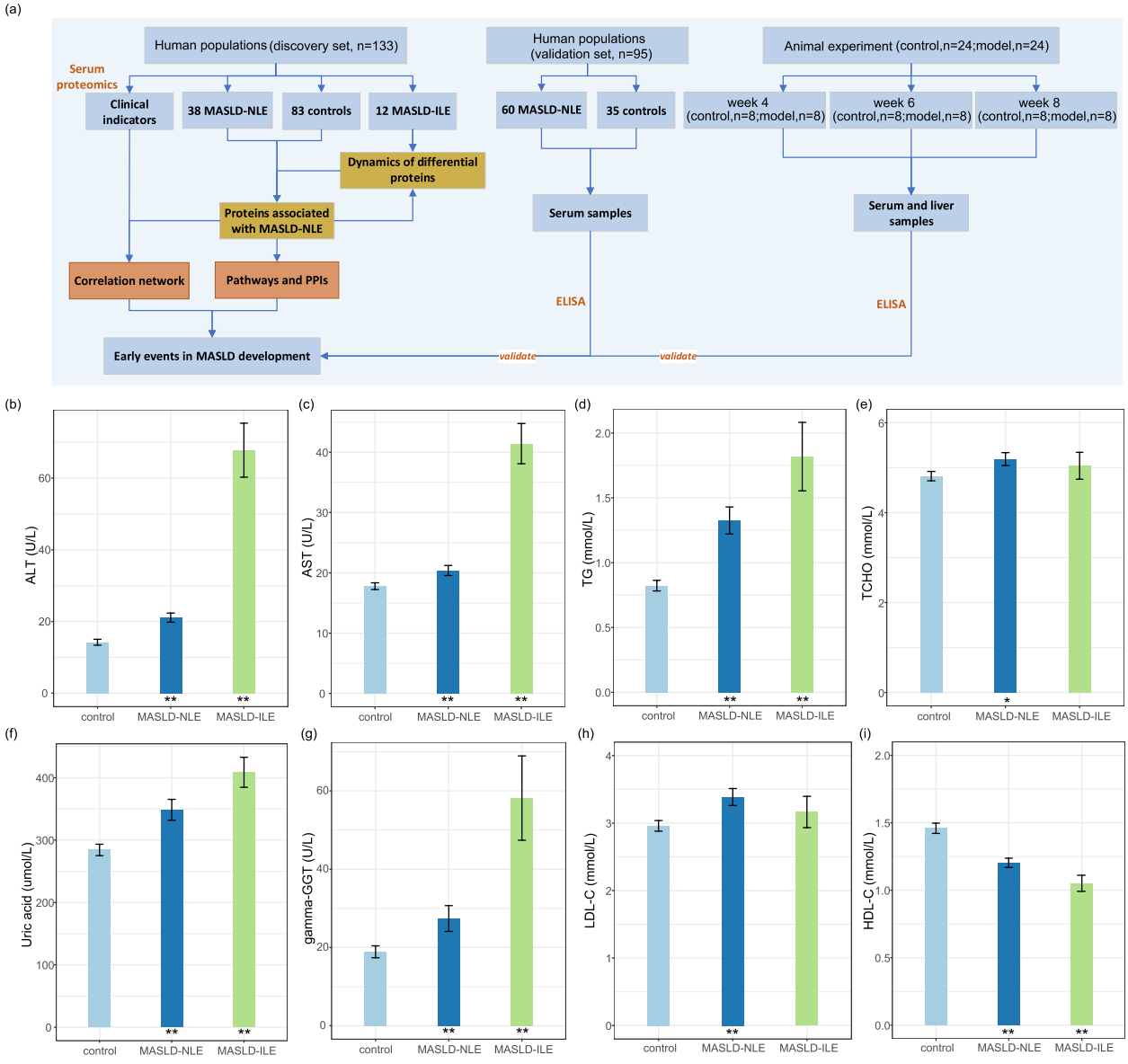

Fig. 1 **a** A schema of study design. Levels of (**b**) ALT, (**c**) AST, (**d**) TG, (**e**) TCHO, (**f**) Uric acid, (**g**) γ-GGT, (**h**) LDL-C, and (**i**) HDL-C in controls, patients with MASLD-NLE and MASLD-ILE. Error bars represent means ± SEs. * and ** represents *P* values less than 0.05 and 0.01, respectively

from patients with MASLD-ILE. Then, we examined the potential roles of the differentially expressed proteins associated with MASLD-NLE in driving the development of MASLD. Another set of serum samples from 35 controls and 60 patients with MASLD-NLE (validation set) was further collected to validate the main findings of the discovery set. Additionally, we induced metabolic dysfunctions in model mice using HFD, and further confirmed the main findings from human studies. The human study was approved by the Medical Ethics Committee of the Affiliated Hospital of Hangzhou Normal University (2021(E2)-KS-049), and written consent was obtained from the participants before enrollment. All animal procedures complied with the guidelines approved by the Committee on the Use of Live Animals in Teaching and Research at the Laboratory Animal Center, Hangzhou Normal University (HSD20220801).

Study participants and sample collection

All patients with MASLD and healthy volunteers in discovery and validation sets were recruited from the Affiliated Hospital of Hangzhou Normal University. The diagnosis of MASLD was based on criteria endorsed by an international expert panel [2, 18]. Concisely, liver steatosis is measured with controlled attenuation parameter (CAP) on an equipped FibroScan. In the presence of hepatic steatosis, the finding of any cardiometabolic risk factors such as BMI ≥ 25 [23 Asia], fasting serum

glucose ≥ 5.6 mmol/L, blood pressure ≥ 130/85 mmHg, and plasma triglyceride ≥ 1.70 mmol/L would confer a diagnosis of MASLD if there are no other causes of hepatic steatosis [2]. Patients with liver steatosis caused by other factors, such as viruses, autoimmunity, genetics, alcohol, or drugs, were excluded from the study. Patients with a history of liver surgery or severe diseases such as renal, liver failure and malignancy were also excluded. The patients with MASLD were further divided into subgroups based on their alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Those with ALT and AST levels below the upper limit of normal (ULN) (40 U/L) were classified as MASLD-NLE, while those with ALT or AST levels exceeding the ULN were classified as MASLD-ILE [19]. Considering that a true healthy normal ALT level ranges from 29 to 33 IU/L for males and 19 to 25 IU/L for females [20], we also utilized an alternative cutoff for normal ALT levels (less than19 and 30 IU/L for females and males, respectively) [21]. For clarity, patients defined by the alternative cutoff were labeled as MASLD-NLE(new) and MASLD-ILE(new). Clinical information was collected at the time of sample collection. Blood samples were obtained from all recruited patients with MASLD and controls after a 12-h fast and with informed consent. Participants were instructed not to take any medications or dietary supplements for 48 h prior to blood collection. The blood samples were centrifuged at 3500 × g for 10 min at 4 °C, and the resulting serum samples were transferred to fresh Eppendorf tubes (subpackaged), and stored at −80 °C until analysis without freeze–thaw cycles.

Serum proteomic analysis

For each sample, 4 μL of serum was initially depleted of 14 high-abundant proteins including albumin, IgA, IgD, IgE, IgG, IgG (light chains), IgM, alpha-1-acid glycoprotein, alpha-1-antitrypsin, alpha-2-macroglobulin, apolipoprotein A1, fibrinogen, haptoglobin, and transferrin using a human affinity depletion resin (Thermo Fisher Scientific™, San Jose, USA). The depleted samples were then concentrated to 50 μL using a 3 K MWCO filtering unit (Thermo Fisher Scientific™, San Jose, USA) following the manufacturer's instructions. As previously described, the resulting serum samples were prepared and utilized for proteomic analysis [22]. All samples were analyzed using a liquid chromatography-coupled tandem mass spectrometer (LC/MS/MS) with a data-dependent acquisition mode on an Orbitrap 480 (Thermo Fisher Scientific, San Jose, CA, USA) using parameters described elsewhere [22]. The resulting spectrometric data were analyzed using Proteome Discoverer (version 2.4.0.305, Thermo Fisher Scientific). A database search was performed using the *Homo sapiens* protein database containing 20,301 reviewed protein sequences downloaded from UniProtKB along with the previously described parameters [22]. The data were normalized based on the total peptide amount. For the 'Total Peptide Amount' mode, it first summed the peptide group abundances for each sample and determined the maximum sum for all files. The normalization factor was the factor of the sum of the sample and the maximum sum in all files.

Differential expression analysis

Proteins that exhibited differential expression between controls and patients with MASLD-NLE were selected according to the $\log_2$ (fold change) ($\log_2$FC > 1.5) and the *P* values (-$\log_{10}$ (*P value*) > 1.3). FC was calculated using the mean values of each protein in each group, while *P* values were calculated using MaAslin2 [23], a method that determines the multivariable associations between MASLD and serum proteins while controlling for fixed effects such as Body Mass Index (BMI), and age. MaAslin2 was selected due to its high performance in preserving statistical power in the presence of repeated measures and multiple covariates, while accounting from the nuances of meta-omics features and controlling false discovery [23]. MaAslin2 was performed using the R package Maaslin2.

Functional enrichment analysis

To perform functional enrichment analysis, which included enriched pathways and protein–protein interaction networks, we utilized STRING [24]. Proteins differentially expressed between control and patients with MASLD-NLE were imported into STRING [24] to identify enriched Gene Oncology (GO) terms and KEGG pathways.

Animal experiment

Healthy eight-week-old male wild-type C57BL/6 J mice (n = 48) weighing 22.13 ± 0.70 g were used in this study to avoid confounding effects of sexual dimorphism in MASLD progression. The mice were purchased from GemPharmatech Co., Ltd (Jiangsu, China), and maintained at the Animal Experiment Center of Hangzhou Normal University. All mice (four mice per cage) were housed under specific pathogen-free (SPF) conditions with a controlled environment (24 ± 2 °C, 12-h light/dark cycle, 40–60 humidity), and had free access to food and drinking water. Based on a preliminary study using 5 animals per group, we calculated the sample size using R packages 'pwr'. It was revealed that the serum ALT level can be observed significantly increased in HFD-fed mice after intervention for 8 weeks with a statistical power 0.9 when 8 mice were involved in each group. After one week of acclimatization, all mice were randomly divided into two groups based on a random number generator: a control group (control, n = 24) (fed a control diet, AIN-93G,

15.8% kcal fat, 63.9% kcal carbohydrate, and 20.3% kcal protein from Dyets) and a model group (model, n = 24) (fed a high-fat diet, AMLN, 40% kcal fat, 40% kcal carbohydrate, and 20% kcal protein, Dyets). During the randomization procedures, the investigators were blinded of outcome assessments. Moreover, all investigators were blinded to group assignments during data collection and analysis. The spatial arrangement of the mice within the same group was randomized. At the end of the 4-week, 6-week and 8-week interventions, following overnight fasting, eight mice in each group were anaesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally and then killed by cervical dislocation [25, 26]. The sample size in each group was determined based on previous researches [27, 28]. Samples of serum and liver were collected, subpackaged, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analyses without freeze–thaw cycles. The levels of ALT and AST in the serum were determined using ALT kits (Nanjing Jiancheng Biotechnology, #C009-2–1) and AST kits (Nanjing Jiancheng Biotechnology, #C010-2–1), respectively.

Histological analysis

Mice liver specimens (approximately 5 mm × 5 mm × 5 mm) were obtained from the right lobe near the portal vein. The specimens were then fixed with 4% (w/v) paraformaldehyde, dehydrated, embedded in paraffin, and sectioned into 5-um-thick slices. Hematoxylin–eosin (H&E; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) staining was performed on the sections following the manufacturer's instructions. The histological liver damage was evaluated using the NAFLD activity score (NAS) system [29], which assesses steatosis, lobular inflammation, and hepatocellular ballooning.

ELISA assays

The levels of C3 (cat#RX201786, RUIXIN BIOTECH, China) and component factor H (CFH) (cat#RX203360, RUIXIN BIOTECH, China) in serum and liver samples were assessed using the corresponding ELISA kits. All reagents, samples, and standards were prepared according to the instructions. The procedure involved the following steps: Transfer 50 µL of each standard or sample to their respective wells; Add 50 µL of Antibody Cocktail to all the wells; Incubate the plate at room temperature for 1 h; Aspirate the contents of each well and wash three times with 350 µL of 1X Wash Buffer PT; Add 100 µL of TMB Development Solution to each well and incubate for 10 min; Finally, add 100 µL of Stop Solution and measure the absorbance at 450 nm. The standards were utilized for quality control, and assessing inter- and intra-assay variability.

Statistical analysis

The univariate clinical data were presented using different measures depending on the variable type. The median and inter quantile range (IQR) were reported for continuous variables, while categorical variables were presented as frequencies. The Wilcoxon rank-sum test assessed differences between groups for univariate variables, and Chi-square test was employed for categorical clinical variables. Permutational multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distance measures were conducted using the R package 'vegan' to examine overall differences in the proteomic compositions between groups, while considering multiple variables simultaneously. Correlation analysis among proteins was performed using the Python module SparCC [30], specifically designed to estimate correlation values from compositional data. Association analysis between proteins and clinical indicators was carried out using Spearman's correlation analysis. Correlations with *P* values after Benjamin-Hochberg correction less than 0.05 were considered statistically significant. Unless otherwise specified, all statistical analyses were conducted using R software (version 4.0.2).

Results

Characteristics of patients with MASLD

Table 1 summarized the median and IQR values for various clinical indices in controls, patients with MASLD-NLE and MASLD-ILE in discovery set. *P* values illustrating the differences between controls and patients with MASLD-NLE for each clinical index were also provided. Surprisingly, despite ALT and AST levels being below the ULN in patients with MASLD-NLE, their levels were significantly higher than those in controls (Figs. 1b-c). Apart from BMI, patients with MASLD-NLE exhibited significantly higher levels of clinical indices such and triglyceride (TG, Fig. 1d), total cholesterol (TCHO, Fig. 1e), hyper-sensitive C reactive protein (hs-CRP), creatine, alkaline phosphatase (ALP), uric acid (Fig. 1f), hemoglobin, and γ-glutamyl transferase (γGGT, Fig. 1g). Furthermore, patients with MASLD-NLE displayed increased levels of low-density lipoprotein cholesterol (LDL-C, Fig. 1h) and decreased levels of high-density lipoprotein cholesterol (HDL-C, Fig. 1i). Then, we utilized an alternative cutoff for normal ALT levels (less than19 and 30 IU/L for females and males, respectively) [21], and re-assessed the median and IQR values for the aforementioned clinical indices in controls and MASLD patients (Table S1). Based on the alternative cutoff, 26 patients were classified as MASLD-NLE(new), and 24 were labeled MASLD-ILE(new). In this case, the same conclusions were reached. Such results indicated that metabolic disorders were already present in patients with MASLD and normal liver enzyme levels.

Table 1 Characteristics of Controls, MASLD-NLE patients (ALT and AST less than 40 U/L), and MASLD-ILE patients (ALT or AST higher than 40 U/L)

	Controls	MASLD-NLE patients	MASLD-ILE patients	Test of significance (MASLD-NLE vs control)
Age (y)	39.00 (26.00, 47.00)	45.00 (38.00, 50.00)	34.50 (29.75, 45.00)	W = 1133.50 (*P* = 0.01)
Sex (male/female)	17/66	22/16	8/4	χ2 = 15.04 (*P* < 0.01)
BMI (kg/m2)	22.07 (20.45, 24.54)	26.37 (25.02, 27.95)	26.91 (24.62, 29.61)	W = 500.00 (*P* < 0.01)
SBP (mmHg)	119.00 (113.00, 127.00)	130.00 (120.25, 137.00)	127.00 (121.50, 135.25)	W = 883.50 (*P* < 0.01)
DBP (mmHg)	73.00 (67.00, 80.00)	80.50 (74.25, 85.75)	83.00 (74.25, 87.50)	W = 953.50 (*P* < 0.01)
Hemoglobin (g/L)	136.00 (132.00, 145.00)	153.00 (138.25, 159.75)	160.50 (141.00, 165.25)	W = 879.00 (*P* < 0.01)
Urid acid (μmol/L)	266.00 (242.50, 301.50)	322.00 (273.75, 398.75)	429.00 (351.25, 462.25)	W = 910.50 (*P* < 0.01)
γGGT (U/L)	14.00 (12.00, 19.00)	22.50 (18.00, 30.50)	42.50 (35.50, 71.50)	W = 816.50 (*P* < 0.01)
DBIL (μmol/L)	4.10 (3.25, 5.10)	3.65 (2.53, 4.58)	4.70 (3.13, 6.33)	W = 1879.50 (*P* = 0.09)
IBIL (μmol/L)	15.40 (12.70, 19.75)	14.40 (11.50, 16.88)	15.20 (11.23, 17.53)	W = 1809.50 (*P* = 0.20)
TBIL (μmol/L)	19.60 (15.70, 23.75)	18.30 (14.55, 20.85)	21.15 (18.13, 22.93)	W = 1839.50 (*P* = 0.14)
ALP (U/L)	62.00 (51.00, 73.00)	70.00 (60.25, 83.25)	74.50 (69.00, 77.25)	W = 1165.50 (*P* = 0.02)
LDL-C (mmol/L)	2.93 (2.53, 3.38)	3.35 (2.84, 3.90)	3.20 (2.69, 3.51)	W = 1066.50 (*P* < 0.01)
HDL-C (mmol/L)	1.43 (1.21, 1.62)	1.20 (1.06, 1.34)	1.06 (0.98, 1.22)	W = 2302.50 (*P* < 0.01)
TCHO (mmol/L)	4.80 (4.22, 5.21)	5.16 (4.56, 5.74)	4.85 (4.35, 5.48)	W = 1162.00 (*P* = 0.02)
TG (mmol/L)	0.750 (0.575, 0.955)	1.15 (0.80, 1.71)	1.52 (1.33, 2.52)	W = 770.00 (*P* < 0.01)
Glucose (mmol/L)	3.86 (3.57, 4.24)	4.05 (3.61, 4.57)	3.78 (3.43, 4.09)	W = 1313.50 (*P* = 0.14)
Alb (g/L)	48.00 (46.55, 49.30)	48.40 (46.63, 50.40)	49.20 (47.75, 49.85)	W = 1405.50 (*P* = 0.34)
hs-CRP (mg/L)	0.51 (0.33, 1.04)	1.09 (0.58, 1.61)	2.21 (1.31, 3.69)	W = 1017.00 (*P* < 0.01)
Creatine (μmol/L)	54.00 (48.50, 60.50)	64.50 (56.00, 76.25)	66.50 (56.75, 74.75)	W = 1023.50 (*P* < 0.01)
ALT (U/L)	11.40 (9.20, 17.30)	19.70 (13.93, 26.65)	56.45 (46.80, 88.00)	W = 712.00 (*P* < 0.01)
AST (U/L)	16.50 (14.35, 21.20)	19.50 (17.60, 23.03)	39.50 (32.95, 46.65)	W = 1059.50 (*P* < 0.01)

BMI body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *γGGT* γ-glutamyl transferase, *DBIL* direct bilirubin, *IBIL* indirect bilirubin, *TBIL* total bilirubin, *ALP* alkaline phosphatase, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TCHO* total cholesterol, *TG* triglyceride, *Alb* albumin;, *hs-CRP* hyper-sensitive C reactive protein, *ALT* alanine transaminase, *AST* aspartate aminotransferase

Perturbation of serum proteins in patients with MASLD-NLE

To probe the proteomic dysregulations associated with MASLD-NLE, we firstly compared the serum proteomic profiles of patients with MASLD-NLE and all controls. The volcano plot revealed that 39 proteins were significantly upregulated, and 15 were downregulated in patients with MASLD-NLE (Fig. 2a). Considering the demographic differences between patients with MASLD-NLE and controls in terms of sample size, age, sex and BMI, we further sampled a subset of 38 controls from the original 83 controls. For the new set of 38 controls (sub-control), they demonstrated comparable sex ratio, age and BMI with those of patients with MASLD-NLE (Figure S1a-c). After re-evaluating the perturbations of the above 54 proteins between sub-controls and patients with MASLD-NLE using Wilcoxon rank-sum test, a final set of 30 proteins were confirmed to be associated with MASLD-NLE. The mean relative abundances of these proteins clearly demonstrated a noticeable distinction between patients with MASLD-NLE and controls before and after resampling (Fig. 2b, Figure S1d). To further assess the differences between controls and patients with MASLD-NLE based on the expression of all differential proteins, we performed principal component analysis (PCA) in both cases (Fig. 2c and Figure S1e). The results from PERMANOVA confirmed a significant perturbation in serum proteins between patients with MASLD-NLE and controls ($P = 0.001$, Fig. 2c), as well as between patients with MASLD-NLE and sub-controls ($P = 0.001$, Figure S1e).

We then conducted correlation analyses to assess the potential cross talks among differentially expressed proteins in controls (Fig. 2d) and patients with MASLD-NLE (Figure S1f), respectively. *P* values and fold change values about the proteins between controls and patients with MASLD-NLE, as well as the correlation networks in controls, were provided in Table S2. Overall, we observed sophisticated yet distinct correlation patterns. For instance, in the control group, C3 showed positive correlations with glutamine synthetase (GLU), CD14, and component factor I (CFI), afamin (AFM), contactin 1 (CNTN1). However, in patients with MASLD-NLE, C3 exhibited positive correlations with attractin (ATRN), lipopolysaccharide-binding protein (LBP), glutathione S-transferase omega 1 (GSTO1), inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4), and negative correlations with apolipoprotein L1 (APOL1), Q92954, transferrin (TF), sex hormone-binding globulin (SHBG). Furthermore, CFH demonstrated positive correlations with inter-alpha-trypsin inhibitor heavy chain 3 (ITIH3), and negative

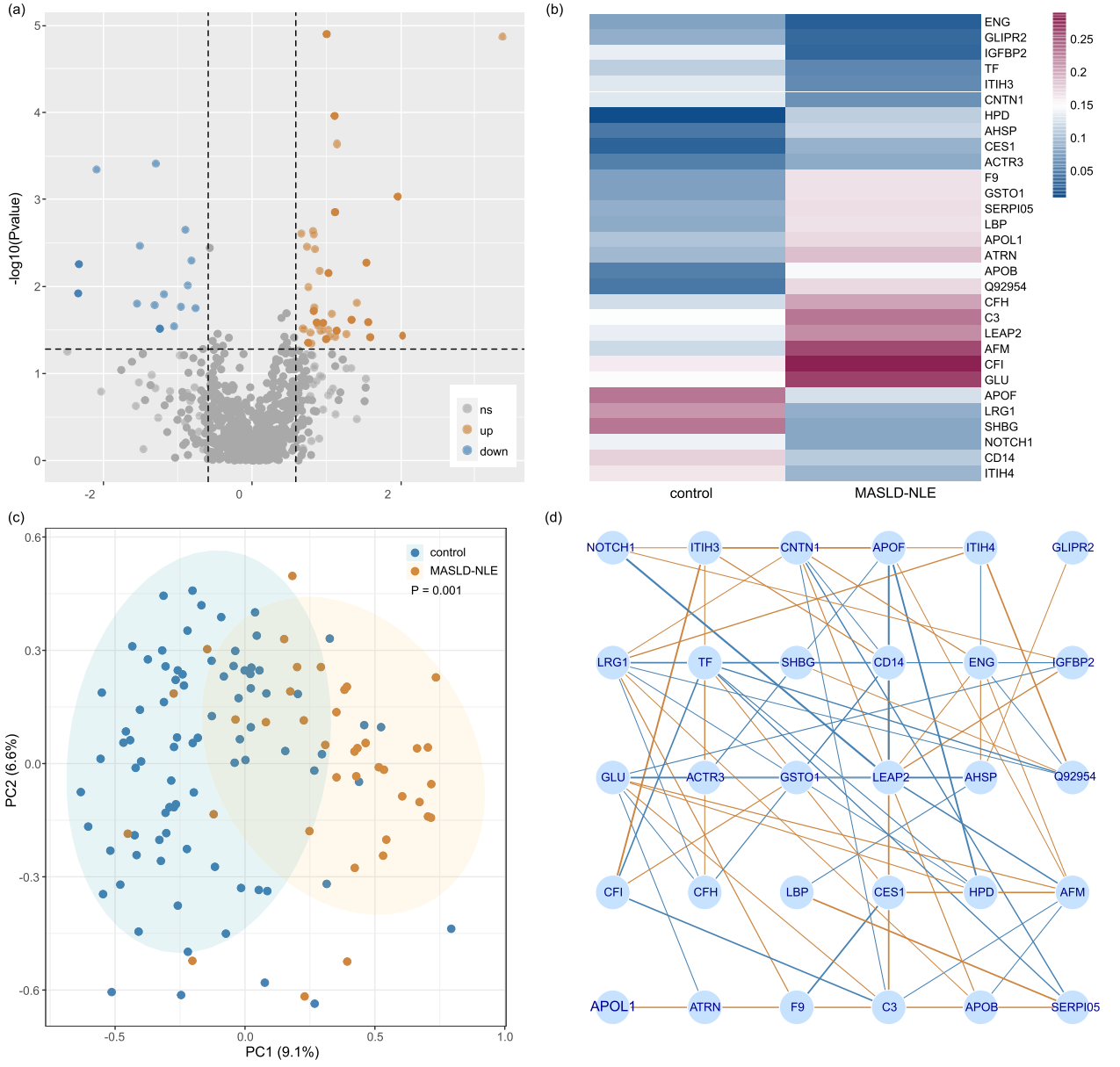

Fig. 2 **a** Volcano plot of serum proteins between healthy controls and patients with MASLD-NLE. **b** Heatmap of differential proteins between controls and patients with MASLD-NLE. **c** The PCA score plot for healthy controls and patients with MASLD-NLE based on differential serum proteins. **d** Associations among differential proteins in controls. Lines colored 'peru' and 'steelblue' represent positive and negative correlations, respectively

correlations with GLU, leucine-rich α2-glycoprotein-1 (LRG1), CD14 in the control group. In patients with MASLD-NLE, CFH exhibited positive correlations with apolipoprotein B (APOB), serpin family A member 5 (SERPI05), actin-related protein 3 (ACTR3), and negative correlations with LBP, SHBG. Additionally, CFI displayed positive correlations with GSTO1, and ITIH3, and negative correlations with C3 and TF in the control group. In patients with MASLD-NLE, CFI demonstrated positive correlations with endoglin (ENG), LBP, LRG1, and negative correlations with Factor IX (F9), GLI pathogenesis related 2 (GLIPR2). In summary, the expression pattern of serum proteins, as well as the potential protein–protein cross talks, was already perturbed in patients with MASLD-NLE. We further compared their relative abundances between patients with MASLD-NLE and MASLD-ILE, with the seven proteins that were differed during MASLD progression shown in Figure S1g. The behavior of all proteins that differ between healthy controls and patients with MASLD-NLE in patients with MASLD-ILE was provided in Table S3.

Elevated complement-related proteins in patients with MASLD-NLE

To understand the molecular events occurring in patients with MASLD-NLE, we conducted functional analyses on the 30 differential proteins. The proteins upregulated in patients with MASLD-NLE were significantly enriched to several KEGG pathways including 'Complement and coagulation cascades' and '*Staphylococcus aureus* infection', as well as Reactome pathways such as 'Regulation of complement cascade', 'Post-translational protein phosphorylation', 'Innate immune system' and 'Immune system' (Fig. 3a). Notably, the KEGG pathway 'complement and coagulation cascades' showed the most significant enrichment with a false discovery rate (FDR) less than 0.0001. We also evaluated the GO terms enriched with the upregulated proteins (Fig. 3b). Besides metabolic pathways 'Regulation of lipid storage', the proteins were most significantly enriched to 'Complement activation, alternative pathway' and 'Complement activation'. We then evaluated the expression of proteins enriched in complement activation, as well as the associations between them and clinical indices. Figure 3c illustrated the elevated expression of proteins C3 and CFH in patients with MASLD-NLE compared to controls. After applying an alternative cutoff for normal ALT levels (less than 19 and 30 IU/L for females and males, respectively) [21] for patients with MASLD, the re-evaluation confirmed the elevated expression of proteins C3 and CFH in patients with MASLD-NLE(new) compared to controls (Fig. 3d, $P < 0.01$). Moreover, the correlation analysis revealed that both C3 and CFH correlated positively with uric acid, γ-GGT, ALP, TG, hs-CRP and ALT, while negatively with HDL-C (Fig. 3e). Such results indicated the activation of complement system in patients with MASLD-NLE, in addition to the presence of metabolic dysfunctions.

Additionally, we evaluated the protein–protein interaction network among the downregulated proteins in patients with MASLD-NLE. Functional enrichment analysis revealed that the downregulated proteins were

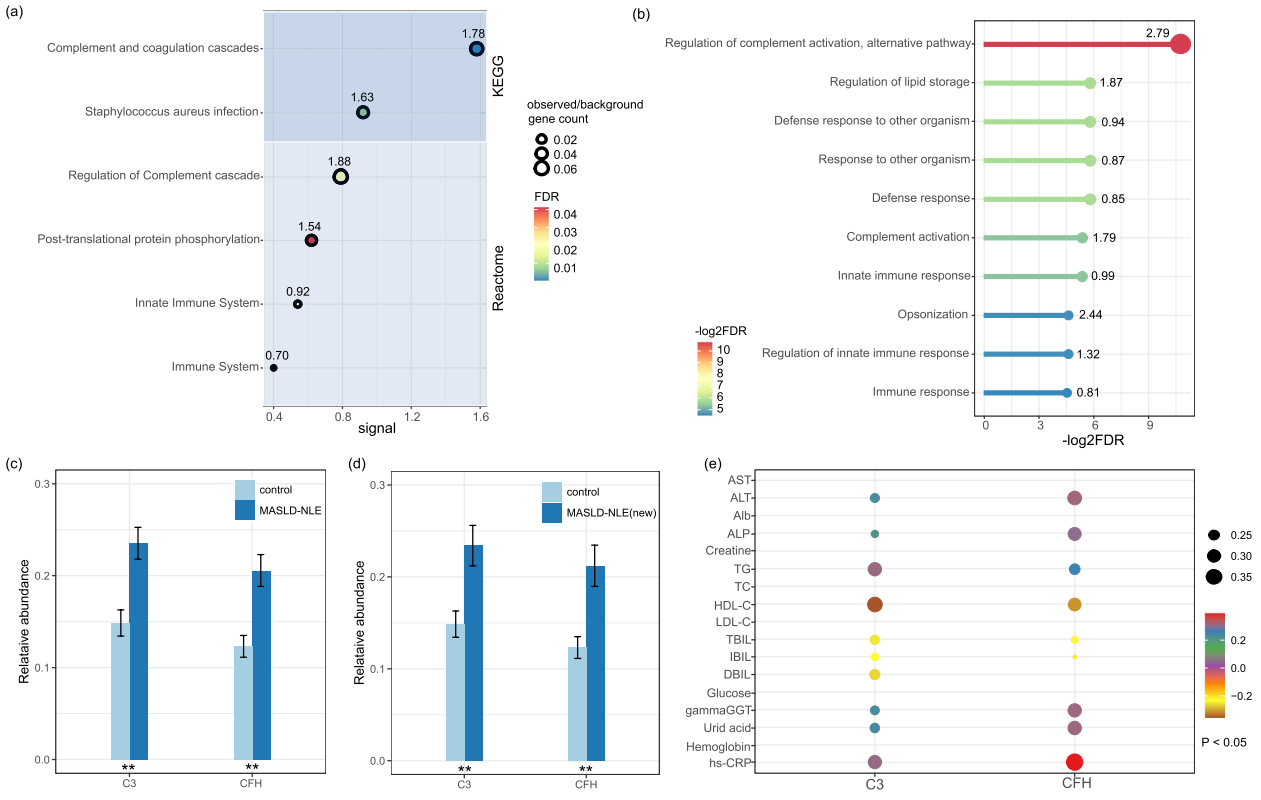

Fig. 3 **a** The KEGG and Reactome pathways enriched by serum proteins upregulated in patients with MASLD-NLE as compared to controls. The signal is defined as a weighted harmonic mean between the observed/expected ratio and -log(FDR). The marked quantitative data measure describes how large the enrichment effect is. It's the ratio between the number of proteins in the network that are annotated with a term and the number of proteins that we expect to be annotated with this term in a random network of the same size. **b** The GO terms enriched by serum proteins upregulated in patients with MASLD-NLE as compared to controls. The marked quantitative data measure describes how large the enrichment effect is. It's the ratio between the number of proteins in the network that are annotated with a term and the number of proteins that we expect to be annotated with this term in a random network of the same size. **c** The relative abundances of proteins C3 and CFH in controls and patients with MASLD-NLE. **d** The relative abundances of proteins C3 and CFH in controls and patients with MASLD-NLE(new). **e** Associations between differential proteins and clinical indicators in controls and patients with MASLD-NLE. ** represents $P < 0.01$

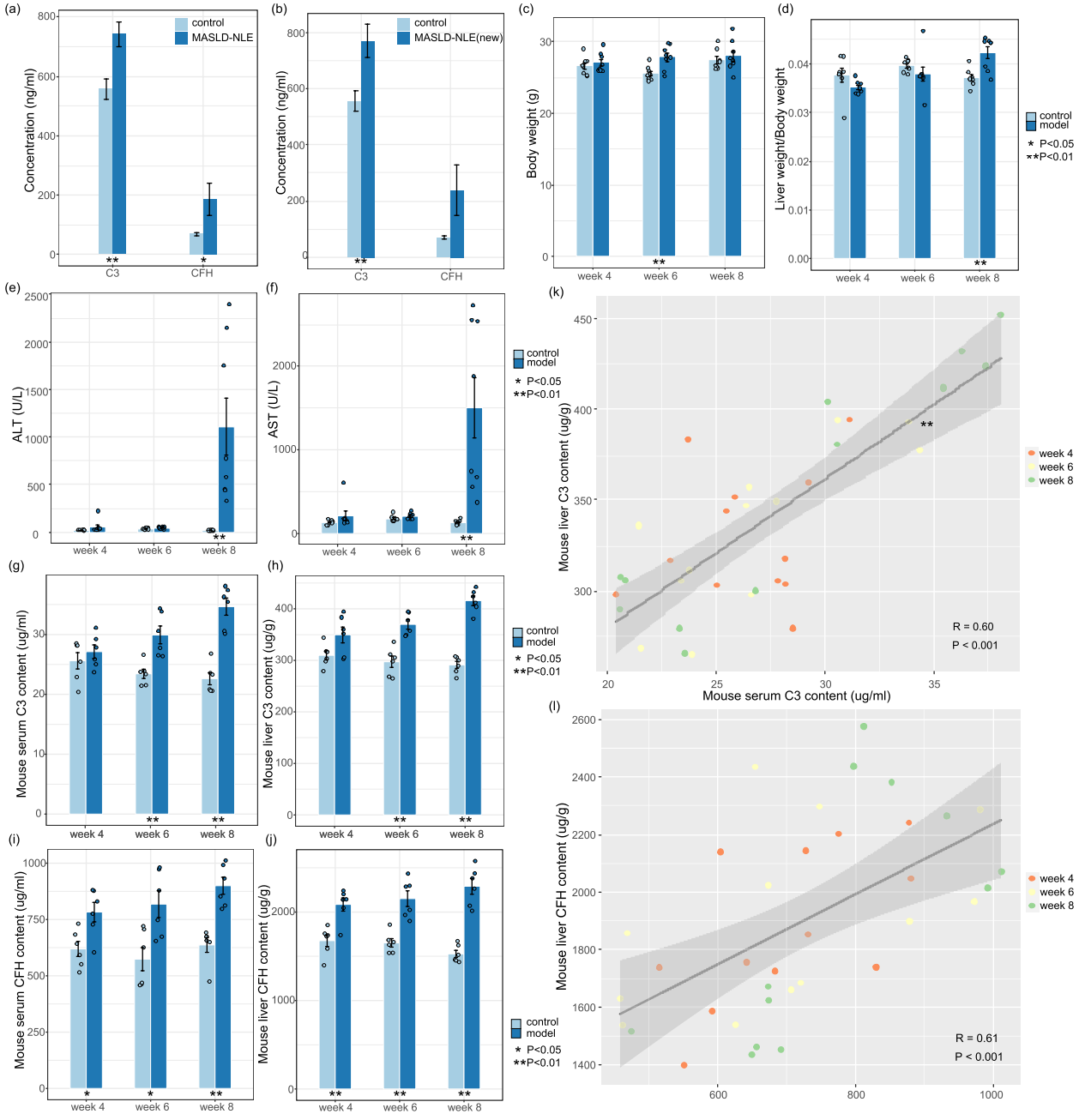

Fig. 4 **a** The concentration of C3 and CFH in controls and patients with MASLD-NLE from validation set. **b** The concentration of C3 and CFH in controls and patients with MASLD-NLE(new) from validation set. **c** Body weight of model mice and corresponding controls at weeks 4, 6 and 8. **d** Ratio of liver weight and body weight for model mice and corresponding controls at weeks 4, 6 and 8. **e** The serum level of ALT in model mice and corresponding controls at weeks 4, 6 and 8. **f** The serum level of AST in model mice and corresponding controls at weeks 4, 6 and 8. **g** The serum level of C3 in model mice and corresponding controls at weeks 4, 6 and 8. **h** The liver C3 contents in model mice and corresponding controls at weeks 4, 6 and 8. **i** The serum level of CFH in model mice and corresponding controls at weeks 4, 6 and 8. **j** The liver CFH contents in model mice and corresponding controls at weeks 4, 6 and 8. **k** The correlations between serum and liver contents of C3 in model mice. **l** The correlations between serum and liver contents of CFH in model mice

enriched in GO terms 'positive regulation of epithelial to mesenchymal transition', and 'Cell migration involved in endocardial cushion formation'. Such results suggested a potential association between MASLD and cardiac disease in its early stages.

Confirmation of elevated complement-related proteins in patients with MASLD-NLE and model mice

The elevation of proteins C3 and CFH in patients with MASLD-NLE and MASLD-NLE(new) were confirmed in a validation set containing serum samples from 35 controls and 60 patients with MASLD-NLE (Fig. 4a and

4b). For further confirmation, we developed a MASLD mice model using HFD and collected serum and liver samples at weeks 4, 6, and 8 during MASLD progression. Figure 4c and 4d presented the body weight, ratio of liver weight to body weight of the model mice and corresponding controls at weeks 4, 6, and 8. Figure 4e and 4f displayed the serum ALT, AST levels. At week 8, the liver to body weight ratio and ALT and AST levels showed significant increases compared to corresponding controls. Figure S2 illustrates the H&E staining results of the liver samples obtained from the control and model mice at weeks 4, 6, and 8, along with corresponding NAS scores. The mouse liver pathology aligned with the serum biochemical results. The mouse liver steatosis was assessed using the NAS and total scores (steatosis, lobular inflammation, and ballooning scores). The NAS score results indicated that only a few mice exhibited liver steatosis at week 4. At week 6, the model mice had an average NAS score of approximately two, representing the MASLD stage predominantly. At week 8, the model mice achieved an average NAS score of approximately 4, meeting the criteria for MASH diagnosis (Figure S2). Combined with the serum ALT levels, these findings indicated that the model mice at week 6 developed MASLD with normal liver enzyme levels (mice-NLE), whereas the model mice at week 8 developed MASLD with increased liver enzyme levels (mice-ILE).

Based on the serum and liver samples obtained from model mice at weeks 4, 6, and 8, we further evaluated the expression of C3 and CFH in serum (Fig. 4g and 4i) and liver samples (Fig. 4h and 4j) using ELISA. The serum and liver contents of C3, and CFH in mice-NLE (week 6) and mice-ILE (week 8) were significantly higher than those in the corresponding controls. Moreover, serum and liver CFH content elevation was significant in the model mice at week 4, and persisted until weeks 6 and 8. Although the elevation of serum C3 content was not as prominent as that at week 4, liver C3 content increased in the mouse model at that time point ($P = 0.09$). Additionally, we observed that the liver contents of C3 and CFH exhibited a positive correlation with their levels in the serum samples (Fig. 4k and 4l). In a word, both the human validation samples and mice model confirmed that, besides metabolic dysfunction, complement-related proteins were elevated in MASLD stage with normal enzyme levels.

Discussion

Based on serum proteomic data from controls, patients with MASLD-NLE and MASLD-ILE, we found that complement-related proteins were elevated in patients with MASLD-NLE. The findings were further confirmed by human serum samples in a validation set, and by serum and liver samples from MASLD mice with normal enzyme levels.

There has been increasing focus on patients with MASLD and elevated liver enzyme levels as they are at a higher risk of developing MASH. However, this study revealed that although the serum levels of ALT and AST were still under ULN in patients with MASLD-NLE, their significant elevation of ALP and γGGT, compared to controls, implied the presence of liver injury in these patients. Hemoglobin, uric acid, and hs-CRP levels also increased prominently in patients with MASLD-NLE. Elevated hemoglobin levels were associated with the incidence of metabolic syndrome [31] and advanced fibrosis in pediatric non-alcoholic fatty liver disease [32]. A higher serum uric acid level was found to be significantly associated with a higher risk of cardiovascular disease (CVD) among patients with MASLD [33], whereas elevated hs-CRP levels were correlated with the severity of MASLD and risk of CVD [34, 35]. Given the significant abnormalities observed in patients with MASLD-NLE, it is crucial to devote more attention to these patients in future studies and clinical treatments. On the other hand, there is still no effective drug treatment for MASLD. Although multiple efforts have shown potential impacts in improving liver histology and biochemical indices such as insulin resistance and liver inflammation in MASLD, especially in MASH patients [36–38], only a minority of patients achieve a treatment response [39]. Furthermore, Elhoseeny et al. found a high frequency of MASLD among young, ostensibly healthy medical students in Egypt, and supported that metabolic dysfunction and MASLD began early, often silently, in a young demographic [40]. Thus, considering the drastic metabolic abnormalities in patients with MASH that are difficult to reverse, developing treatment options for patients with MASLD-NLE to delay and/or prevent MASLD progression may be a better solution. In this situation, multiple studies have demonstrated that the presence of an elevated ALT has been associated with increased liver-related mortality [20]. Moreover, the degree of elevation of ALT and or AST in the clinical setting helps guide the evaluation. For example, minimal elevations may be approached by a history and examination, discontinuation of hepatotoxic medicines, all alcohol assumption, as well as an evaluation of hemochromatosis, fatty liver and viral hepatitis [20]. Higher degrees of elevation (5–15 times the upper limit) should be approached with an evaluation of viral hepatitis A, B, and C, autoimmune markers and so on [20]. Thus, a true healthy normal ALT levels ranging from 29 to 33 IU/l for males, 19 to 25 IU/l for females proposed in ACG clinical guidance might be more appropriate for clinical applications.

Complement activation has been reported in human MASLD [41]. However, we reported for the first time that

complement-related proteins were elevated in patients with MASLD-NLE. This finding was confirmed using human serum samples from a validation set, as well as serum and liver samples from MASLD mice with normal liver enzyme levels. The complement system is traditionally considered a component of innate immunity [42, 43], but it is now recognized as a central component of innate and adaptive immunity. The complement system plays a key role in the development of MASLD, with particular attention given to the component C3. Complement activation through the classical, alternative, and lectin pathways converges in the formation of fraction C3. Higher serum C3 levels were associated with MASLD, and patients with MASLD and higher serum C3 levels were more likely to have increased liver enzyme levels [41, 44]. Histological studies have confirmed the link between the component system and MASLD, showing the deposition of activated forms of C3 in most MASLD patients. Furthermore, MASH is more prevalent in activated C3-positive patients [41]. These results suggest a positive relationship between complement activation and the prevalence and severity of MASLD, including MASH [45, 46]. However, patients with MASLD exhibit high heterogeneity due to differences in their natural histories and interindividual disease courses. The initiation of complement activation during MASLD progression remains unknown. The discovery that complement-related proteins were elevated in patients with MASLD-NLE fills a critical gap in the association between complement activation and MASLD, and it may serve as a foundation for developing novel therapeutic strategies that target the complement system in early stages of MASLD progression.

Serum fatty acids, adipose tissue-derived cytokines and gut derived endotoxin are reported to take part in complement activation [47]. Hepatocytes are confirmed to be the predominant origin of complement components, although adipose tissue can also produce certain components, such as C3 [48]. This study revealed that there was a significant correlation between the C3 and CFH in serum and liver samples. These findings suggest that the serum levels of C3 and CFH in patients with MASLD-NLE and MASLD mice are likely derived from the liver with a high probability. Nowadays, the underlying mechanism by which complement C3 in NAFLD remained elusive. However, C3 is hypothesized to play an important role in regulating lipid metabolism and the oxidative stress in hepatocytes with excessive fat accumulation. The large number of inflammatory cytokines secreted in the liver due to the response of hepatocytes to oxidative stress, further causes the second hit in hepatocytes [49]. Then, activated complement system immediately recognizes and cleans the apoptotic pathological liver cells, thus maintaining the homeostasis in liver [50]. In brief, there is a potential balance in the pathogenesis of NAFLD between complement system activation and hepatocyte lipid metabolism signaling, which maintains the stability of liver internal environment. C3 was reported to contribute to the accumulation of triglycerides in the liver and adipose tissue of alcoholic fatty liver disease (ALD) [51]. Hepatitis B virus can downregulate the synthesis and secretion of C3 and C4 both in vitro and in vivo [52], while C3 knockout led to accelerated hepatic triglyceride accumulation and impaired lipophagy in hepatocytes [53]. Increased production of C3 and C4 in hepatocytes was also observed in patients with autoimmune hepatitis, although the roles of complement was limited [54]. Such results further indicated the importance of balanced complement system in liver diseases. Once the balance is broken, the process of hepatocyte lipid metabolism will enter a vicious circle, which exacerbates irreversibly hepatic steatosis as a consequence. Actually, our study found that serum C3 levels in patients with MASLD-NLE exhibited a significant correlation with clinical features, such as γGGT, hs-CRP, uric acid and ALT. Complement C3 was found to promote islet β-cell dedifferentiation via activation of Wnt/β-catenin pathway, a key step in the progression of diabetes [55]. Uric acid can stimulate the expression of C-reactive protein and complement C3 in a dose-dependent fashion, and finally induced the expression of hepatic inflammatory molecules by activating the NF-κB signaling cascades [56]. In other words, in patients with MASLD-NLE, the elevation of serum complement C3 might act as a protective response with a delicate balance. Such results indicated that targeting the complement system in the liver might be a potential early treatment option for MASLD. Nowadays, complement-targeted therapies have demonstrated safety and efficacy in conditions characterized by hemolysis and certain autoimmune diseases [57], ongoing researches and developments in this field are still crucial for the advancement of therapies to broader applications, since the pathogenic mechanisms of diseases such as MASLD and autoimmune diseases are multifaceted and quite complex, and any dysregulation of the complement system may lead to a spectrum of diseases, as well as individual differences [41]. In a word, further studies with longitudinal design and more validated patients with MASLD-NLE are still required to confirm the findings of this study. Considering the heterogeneity of MASLD patients, subtype analysis of patients with MASLD-NLE is also required.

Conclusion

In summary, we reported that although ALT and AST levels were within the UNL, patients with MASLD-NLE were accompanied by a series of metabolic abnormalities that should not be overlooked clinically. Moreover, we revealed for the first time that the complement-related

proteins were already elevated in patients with MASLD-NLE, which we further confirmed using human serum samples from a validation set, as well as serum and liver samples from MASLD mice with normal liver enzyme levels. The significant correlations between C3 and clinical indices such as ALT, hs-CRP, and uric acid, as well as between serum and liver contents of C3 and CFH, further implied the potential value of targeting complement activation in the liver for the early treatment of MASLD. Although further studies are required to characterize detailed aspects of the early stage of MASLD, our study paves the way for the development of optimal clinical treatment and management protocols aimed at early disease control.

Abbreviations

ACTR3	Actin-related protein 3
ADH1A	Alcohol dehydrogenase 1a
AFM	Afamin
ALP	Alkaline phosphatase
ALDOB	Fructose-biphosphate aldolase
ALT	Alanine aminotransferase
APOB	Apolipoprotein B
APOF	Apolipoprotein F
APOL1	Apolipoprotein L1
ARHGDIA	Rho GDP dissociation inhibitor alpha
AST	Aspartate aminotransferase
ATRN	Attractin
BMI	Body mass index
CAP	Controlled attenuation parameter
CFH	Complement factor H
CFI	Complement factor I
CNTN1	Contactin 1
DBP	Diastolic blood pressure
ENG	Endoglin
EVs	Extracellular vesicles
FAH	Fumarylacetoacetate hydrolase
FN3KRP	Fructosamine 3 kinase-related protein
F9	Factor IX
GLIPR2	GLI pathogenesis related 2
GLU	Glutamine synthetase
GO	Gene oncology
GPLD1	Glycosylphosphatidylinositol specific phospholipase D1
GSTO1	Glutathione S-transferase omega 1
HDL	High-density lipoprotein
HFD	High-fat diet
HPD	4-hydroxyphenylpyruvate dioxygenase
hs-CRP	High sensitivity C reactive protein
IDH1	Isocitrate dehydrogenase
IQR	Interquartile range
IR	Insulin resistance
ITIH3	Inter-alpha-trypsin inhibitor heavy chain 3
ITIH4	Inter-alpha-trypsin inhibitor heavy chain 4
LBP	Lipopolysaccharide-binding protein
LRG1	Leucine-rich α2-glycoprotein-1
MASLD	Metabolic dysfunction-associated steatotic liver disease
MASLD-ILE	Patients with MASLD and increased liver enzyme levels
MASLD-NLE	Patients with MASLD and normal liver enzyme levels
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
PERMANOVA	Permutational multivariate analysis of variance
PON3	Paraoxonase 3
SERPI05	Serpin family A member 5
SBP	Systolic blood pressure
SHBG	Sex hormone-binding globulin
TCN2	Transcobalamin 2
TF	Transferrin
TG	Triglyceride
ULN	Upper limit of normal
γGGT	γ-Glutamyl transferase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12876-025-04361-5.

Additional file 1: Figure S1. (a) Sex ratio of patients with MASLD-NLE and sub-controls. (b) Age of patients with MASLD-NLE and sub-controls. (c) BMI of patients with MASLD-NLE and sub-controls. (d) Heatmap of differential proteins between sub controls and patients with MASLD-NLE. (e) The PCA score plot for sub-controls and patients with MASLD-NLE based on differential serum proteins. (f) Associations among differential proteins in patients with MASLD-NLE. Lines colored 'peru' and 'steelblue' represent positive and negative correlations, respectively. (g) The relative abundances of serum proteins that varied significantly between healthy controls and MASLD-NLE patients, as well as between MASLD-NLE and MASLD-ILE patients.

Additional file 2: Figure S2. (a) The H&E results of liver samples obtained from control and model mice at weeks 4, 6 and 8. The (b) NAS score and NAS sub-scores including (c) steatosis, (d) lobular inflammation, and (e) hepatocellular ballooning for control and model mice at weeks 4, 6 and 8. * and ** represent ***P*** values less than 0.05 and 0.01, respectively.

Additional file 3: Table S1. Characteristics of Controls, patients with MASLD-NLE(new) (ALT less than 30 and 19 U/L for males and females, respectively), and patients with MASLD-ILE(new) (ALT higher than 30 and 19 U/L for males and females, respectively).

Additional file 4: Table S2. ***P*** values and fold change values about the proteins between controls and patients with MASLD-NLE, as well as the correlation networks in patients with MASLD-NLE.

Additional file 5: Table S3. The behavior of all proteins that differ between healthy controls and patients with MASLD-NLE in patients with MASLD-ILE.

Acknowledgements

This work was supported by the key medical disciplines of Hangzhou and the Interdisciplinary Research Project of Hangzhou Normal University (2024JCXK06). The authors thank all the participants who helped collecting serum samples and clinical information.

Authors' contributions

L.S. conducted formal analysis and wrote the manuscript. B.Z. conducted animal experiment. J.L., Z.L., J.F., W.Z. and X.Z. enrolled patients and collected serum samples. J.W. conducted ELISA experiment. W.Y. conducted histological evaluation. J.S. and J.L. conceptualized and revised the manuscript. All authors reviewd the manuscript.

Funding

The present study was funded by grants from the Natural Science Foundation of Zhejiang Province (LY22C010001, GF20H030035, LQ19H030002, LQ23H270016, LHDMZ24H030001), the National Natural Science Foundation of China (31870839, 82170609, 81970545, 82204827), the China University Industry-University-Research Innovation Fund (2022BC158), and the Noncommunicable Chronic Diseases-National Science and Technology Major Project (2023ZD0508704). None of the funding sources play any roles in any aspect pertinent to the study.

Data availability

The data that supports the findings of this study can be provided upon reasonable request.

Declarations

Ethics approval and consent to participate

This human study was approved by the Medical Ethics Committee of the Affiliated Hospital of Hangzhou Normal University (2021(E2)-KS-049) and was

conducted according to the principles of the Helsinki Declaration. Written informed consent was obtained from all participants before enrollment in the study. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at the Laboratory Animal Center, Hangzhou Normal University (HSD20220801, Hangzhou, Zhejiang, China).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 3 July 2025 / Accepted: 26 September 2025
Published online: 29 October 2025

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