Phenotypic and Metabolomic Characteristics of Mouse Models of Diet-Induced Hepatic Steatosis

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Abstract

Background

Nonalcoholic steatohepatitis (NASH) is a metabolic disease that may progress to cirrhosis and hepatocellular carcinoma. Mouse models of diet-induced NASH, which is characterized by the high levels of fats, sugars, and cholesterol in diets, are commonly used in research. However, mouse models accurately reflecting the progression of NASH in humans remain to be established. Studies have explored the potential use of serological metabolites as biomarkers of NASH severity in relation to human NASH.

Methods

We performed a comparative analysis of three mouse models of diet-induced NASH in terms of phenotypic and metabolomic characteristics; NASH was induced using different diets: a high-fat diet; a Western diet; and a high-fat, high-cholesterol diet. Liver cirrhosis was diagnosed using standard clinical approaches (e.g., METAVIR score, hyaluronan level, and collagen deposition level). Mouse serum samples were subjected to nuclear magnetic resonance spectroscopy–based metabolomic profiling followed by bioinformatic analyses. Metabolomic analysis of a retrospective cohort of patients with hepatocellular carcinoma was performed; the corresponding cirrhosis scores were also evaluated.

Results

Using clinically relevant quantitative diagnostic methods, the severity of NASH was evaluated. Regarding metabolomics, the number of lipoprotein metabolites increased with both diet and NASH progression. Notably, the levels of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) significantly increased with fibrosis progression. During the development of diet-induced NASH in mice, the strongest upregulation of expression was noted for VLDL receptor. Metabolomic analysis of a retrospective cohort of patients with cirrhosis indicated lipoproteins (e.g., VLDL and LDL) as predominant biomarkers of cirrhosis.

Conclusions

Our findings provide insight into the pathophysiology and metabolomics of experimental NASH and its relevance to human NASH. The observed upregulation of lipoprotein expression reveals a feedforward mechanism for NASH development that may be targeted for the development of noninvasive theranostics.

Background
Nonalcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease worldwide. NAFLD is a progressive condition characterized by excess fat accumulation in the hepatocytes of individuals who consume very little or no alcohol.[1, 2] This disease is commonly associated with unhealthy lifestyle, obesity, type 2 diabetes, insulin resistance, dyslipidemia, and metabolic syndrome.[1, 3, 4] NAFLD can develop as hepatic steatosis or severe conditions involving inflammation, such as nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC), and liver failure.[3, 4] NASH is characterized by hepatic inflammation, hepatocyte damage, and intrahepatic fat accumulation.[5, 6] The resultant inflammation and damage lead to liver fibrosis and cirrhosis—a severe, irreversible condition. Patients with NASH are at a higher risk of liver cancer, liver failure, and cardiovascular disease than are healthy individuals.[2, 5] Histologically, NASH is characterized by steatosis, inflammation, hepatocyte ballooning and some with or without fibrosis. Key risk factors for NASH include diabetes, obesity, age, ethnicity, sex, and genetic polymorphisms, which can also affect NASH progression.[7, 8] These risk factors reflect the complex and heterogeneous nature of NASH.[9] Although extensive studies have been conducted on NAFLD and NASH, the precise mechanisms underlying the development of NAFLD and its progression to NASH have yet to be elucidated.

Several genetic and dietary models of NASH have been developed using various research animals. These models mimic human NASH in terms of its pathophysiological characteristics, including the presence of obesity, insulin resistance, and dyslipidemia.[10, 11] The most commonly used model for studying diet-induced NAFLD is the inbred C57BL/6 mouse model.[11, 12] Diets used for inducing NASH include high-fat diets (HFDs) and methionine- and choline-deficient diets.[13, 14] These experimental diets as well as different diet types and feeding lengths induce NAFLD/NASH with varying degrees of severity.[10, 12]

The calorie intake of HFDs with a high fat content (45–75%).[10, 15] Feeding animals an HFD can induce NAFLD mimicking the major histopathological and pathogenic features of human NAFLD. Long-term intake of an HFD may induce obesity, insulin resistance, and hepatic damage in animal models.[15, 16] Although HFD-induced experimental NAFLD can mimic the altered metabolic pattern observed in human NAFLD, experimental NAFLD cannot progress to a severe hepatic pathology. To closely mimic human NASH, animals are sometimes fed an HFD supplemented with other additives, such as fructose and cholesterol.[17, 18]

High cholesterol intake can induce dyslipidemia and insulin resistance and is a crucial factor associated with hepatic inflammation and NASH progression in both animal models and humans.[15, 19–21] Elevated levels of cholesterol can increase hepatic oxidative stress and promote hepatocyte apoptosis, macrophage recruitment, and fibrogenesis, thus accelerating NASH progression.[15] Fructose can promote de novo lipogenesis, inhibit β-oxidation, and induce insulin resistance in the liver, resulting in the intrahepatic accumulation of lipids, which leads to hepatocellular damage; thus, the supplementation of HFDs with fructose accelerates NASH progression through increased inflammation and fibrosis in mice.[22, 23] However, HFDs supplemented with fructose do not consistently lead to advanced fibrosis in C57BL6/J mice.[23, 24]
Because experimental diet-induced NASH models are developed to mimic the pathophysiology of human NASH, obtaining a detailed account of serological markers is essential. However, few studies have focused on this topic. A comparative analysis of NASH induced by different diets in terms of the changes in serological metabolites is necessary; in addition, the likely association between metabolomic profile and NASH severity must be investigated. Therefore, we conducted the present study to compare three mouse models of diet-induced NASH/cirrhosis in terms of phenotypic and metabolomic characteristics. NASH was induced using different diets: an HFD; a Western diet (WD; high-fat, high-fructose diet); and a high-fat, high-cholesterol diet (HFC). Clinically relevant quantitative diagnosis was performed to evaluate NASH severity in mice. Nuclear magnetic resonance (NMR) spectroscopy–based metabolomic profiling was performed using serum samples obtained from the experimental mice. The acquired data were subjected to bioinformatic analyses to evaluate the NASH severity and phenotypic progression.

Material & Methods

Animal diets and experimental design

Thirty male C57BL/6J mice (age, 7 wks) were purchased from the National Laboratory Animal Center and were housed individually. After 1 wk of adaption and quarantine, the mice were divided into three groups on the basis of diet: an HFD group (the mice obtained 60% of their total energy from fat; Cat#: 58Y1; Young Li Trading Co., Ltd., Taiwan), a WD group (the mice obtained 39.9% of their total energy from fat and 44.1% of their total energy from carbohydrates [fructose]; Cat#: 5TJN; Young Li Trading Co., Ltd., Taiwan), and an HFC group (the mice obtained 39.4% of their total energy from fat and 2% of their total energy from cholesterol; Cat#: 5S8X; Young Li Trading Co., Ltd., Taiwan). The mice were fed experimental diets for 16 and 32 wks to induce NAFLD/NASH with varying degrees of severity. At the end of the feeding program, the mice were euthanized, and their liver specimens and blood samples were collected for further analysis. Through cardiac puncture, blood samples were collected into ethylenediaminetetraacetate-coated tubes and subsequently centrifuged at 1000 × g for 10 min at 4°C. The collected liver specimens were cut into thin sections (0.5 cm × 0.5 cm), fixed in 10% formalin, and subjected to paraffin embedding. The protocols for the animal experiments were approved by the Animal Ethics Committee of China Medical University (approval number: CMUIACUC-2021-061).

Real-time quantitative polymerase chain reaction

Total RNA was extracted from the liver tissues of the mice by using TRIzol (Cat: T9424, Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions.[25] The obtained RNA (5 µg) was reverse-transcribed using the PrimeScript RT Reagent Kit (Cat: RR037A; TaKaRa, Tokyo, Japan) according to the manufacturer's instructions.[26] Quantitative polymerase chain reaction (PCR) was performed using the KAPA SYBR FAST qPCR Master Mix (KM4100; KAPA Biosystems) with specific primers (see supplementary Table 1); for this, the Azure Cielo Real-Time PCR System (Azure Biosystems, Dublin, CA, USA) was used. Gene expression levels were normalized against the expression level of actin, and the relative changes in gene expression were quantified using the \(2^{-\Delta\Delta Ct}\) method.
Detection of hyaluronan through sandwich enzyme-linked immunosorbent assay

The presence of hyaluronan in the serum samples of the experimental mice was detected through sandwich enzyme-linked immunosorbent assay (ELISA). All blood samples were centrifuged at 1000 \( \times g \) for 15 min at 4°C; the obtained plasma samples were subjected to sandwich ELISA, which was performed using Quantikine ELISA Kits (Cat#: DHYAL0; R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. In brief, all reagents, working standards, and samples were prepared as directed. To each well, 50 µL of Assay Diluent RD1-14 was added followed by 50 µL of the standard, control, or sample. The ELISA plate was incubated on a horizontal orbital shaker (70 rpm) for 2 h at 25°C. After incubation, each well was aspirated and washed five times with 400 µL Wash Buffer. Next, 100 µL of hyaluronan conjugate was added to each well. The plate was covered with a clean adhesive strip and incubated on the shaker for 2 h at room temperature. After incubation, the wells were aspirated and washed as indicated. Subsequently, 100 µL of substrate solution was added to each well. The plate was incubated in the dark for 30 min at room temperature. After incubation, 100 µL of stop solution was added to each well. Within 30 min of the addition of the stop solution, absorbance was measured at 450 and 570 nm by using a microplate reader; for background correction, the readings obtained at 570 nm were subtracted from those obtained at 450 nm.

Histopathological analysis

Paraffin-embedded liver tissue sections were stained with hematoxylin–eosin (H&E) and Masson’s trichrome[27] for histopathological analysis. Using the METAVIR scoring system,[28] a licensed pathologist who was blinded to the group allocation scored the Masson's trichrome–stained liver tissues for fibrosis and cirrhosis. Collagen fibers were detected in the tissue sections stained with Masson's trichrome. Tissue images were captured at 20× magnification. The area containing collagen fibers was assessed using ImageJ.[29] Collagen deposition was quantified using 20 images per liver sample.

NMR spectroscopy–based metabolomic profiling

A retrospective cohort of patients with cirrhosis was analyzed. Serum samples were collected from 80 patients with HCC and subjected to NMR spectroscopy–based metabolomic profiling by using the Nightingale Health platform (Helsinki, Finland).[30] This platform facilitates the simultaneous detection of 151 serum biomarkers and provides a comprehensive spectrum of metabolites. The biomarkers include lipid metabolites, such as cholesterol, triglycerides, various fatty acids, apolipoprotein (Apo)A1, and ApoB; amino acid; glycolysis-related metabolite; ketone bodies; creatinine; albumin; and glycoprotein acetyl. The size, subclass distribution, and loading lipids of lipoprotein metabolites can also be analyzed using this platform. Details regarding the observed serological metabolites, including their abbreviations and units, are presented in Supplementary Table 2.

For metabolomic profiling, mouse plasma samples were assessed through NMR spectroscopy (Ascend 600C) on the Burker high-throughput metabolomics platform. The standardized platform included Bruker
IVDr Lipoprotein Subclass Analysis (model version: PL-5009-01/001) and the automated quantification of small metabolites (model version: Quant-PS 2.0.0). Details regarding the metabolites, including their abbreviations and units, are presented in Supplementary Tables 3 and 4.

Retrospective analysis of patients with fibrosis or cirrhosis

A total of 113 patients who received a confirmed diagnosis of HCC between 2009 and 2013 at China Medical University Hospital were retrospectively included in this study. Patients with missing liver biopsy or key hematological data; those with blood samples unsuitable for metabolite quantification; those aged < 18 or > 80 years; those receiving long-term drug therapy; and those with advanced cancer metastasis, HIV infection, autoimmune disease, or other liver-related comorbidities (e.g., Wilson's disease, haemochromatosis, alpha-1 antitrypsin deficiency, lupoid hepatitis, and cholestatic or vascular liver disorders) were excluded from this study. Finally, 80 patients were included in the analysis. The patients’ demographic and medical data were recorded by trained research assistants. The effects of the following covariates were adjusted in the models used for statistical analysis: age (years), sex (male or female), body mass index (kg/m$^2$), smoking status (yes [smoked for at least 1 year] or no), hypertension (blood pressure of > 140/90 mmHg or previous physician diagnosis), type 2 diabetes (fasting glucose level of > 126 mg/dL or previous physician diagnosis), hepatitis B surface antigen (yes or no), hepatitis C antibody (yes or no), Child–Pugh score (A + B or C), ascites (yes or no), hepatic steatosis (yes or no), tumor size (cm), cancer stage (I + II or III + IV), microscopic venous invasion (yes or no), macroscopic venous invasion (yes or no), lymph node involvement (yes or no), and capsule (yes or no) and satellite nodules (yes or no). This study was approved by the Ethics Committee of China Medical University. Written informed consent was obtained from all patients.

Bioinformatic analyses

For metabolite analysis, all data were subjected to normality tests (Shapiro–Wilks and Anderson–Darling tests) and Q–Q plot analysis. Among the metabolites, > 50% were abnormally distributed; therefore, subsequent analyses were performed using nonparametric tests. The metabolites were divided into three groups: lipoproteins and small metabolites, lipoprotein metabolites, and small metabolites.

The metabolite groups were subjected to principal component analysis (PCA). Hierarchical clustering heatmaps were independently constructed using R package gplots. Before analysis, each feature was scaled to have a mean value of 0 and a standard deviation value of 1.

Multiple comparisons were performed using the Kruskal–Wallis test in different groups of diets. Statistical significance was set at $p < 0.05$. To analyze the differentially expressed metabolites, the Wilcoxon rank-sum test was performed, with fold changes and adjusted $p$ values calculated for between-group comparisons. Metabolites were considered to have significance when the corresponding $p$ values were < 0.05 and log2 fold changes were greater than or equal to 1 or less than or equal to −1. Spearman correlation analysis was performed to investigate the correlation between collagen scores...
and metabolites. A $p$ value of $< 0.05$ and a correlation coefficient of $> 0.3$ indicated statistical significance.

For the cohort of patients with cirrhosis, between-group comparisons of metabolites were performed using a two-tailed Student's $t$ test. The resulting $p$ values were adjusted for multiple comparisons by using the Benjamini–Hochberg correction method.\cite{41, 42} Statistical significance was set at a $q$ value of $< 0.05$. Significant metabolites were selected to construct a Pearson correlation matrix,\cite{43} which was subsequently subjected to hierarchical cluster analysis, in which the Euclidean distance\cite{44} was measured using the average method. All statistical analyses were performed using R (version 4.1.0).

**Statistical analysis**

The study data are presented in terms of mean ± standard deviation values and were analyzed with GraphPad Prism (version 8.0). The unpaired Student's $t$ test was used for between-group comparisons. A $p$ value of $< 0.05$ was considered to be significant.

**Results**

**Phenotypic characteristics of mouse models of diet-induced NASH**

Mice fed a normal chow (NC) diet were used as the control group. Figure 1A presents the nutritional composition of each diet and the percentage of total energy contributed by each dietary component. The feeding schedule is depicted in Fig. 1B. The changes in the body weights (BW) of the mice in the three experimental diet groups were recorded. A rapid increase was noted in the BW of all three groups; however, measurements performed when the mice were aged 24 wks revealed that BW gain was faster in the HFD and WD groups than in the HFC group (Fig. 1C). After 16 and 32 wks, the livers were larger and lighter (in color) in the experimental groups than in the control group (Fig. 1D).

The ratio of liver weight (LW) to BW is a robust and vital indicator of liver homeostasis. Normally, the LW/BW ratio ranges from 4.5–5%. Changes in the LW/BW ratio indicate disease progression. After 16 wks of feeding, we noted no significant change in the LW/BW ratio in the four (HFD, WD, HFC, and NC) groups. By contrast, after 32 wks of feeding, the ratio increased significantly in the three experimental groups compared with the ratio in the control group (Fig. 1E). Thus, prolonged intake of the experimental diets led to pathological changes in the mouse liver that accelerated diet-induced NAFLD/NASH. Notably, BW gain was slower and the LW/BW ratio was higher in the HFC group than in the other groups; this indicates a more rapid progression of NASH in the HFC group than in the HFD and WD groups. In summary, all three experimental diets induced NAFLD/NASH with varying degrees of severity.

**Importance of clinically relevant diagnostic methods for the severity of diet-induced NASH in mice**
Clinically relevant quantitative diagnosis was performed to evaluate the severity of diet-induced NASH. The results of histopathological staining performed using the liver tissue samples of each group are presented in Fig. 2A. The results of H&E staining revealed no abnormalities in the control group but gradual pathological changes (e.g., hepatic steatosis and hepatocyte ballooning; after 16 and 32 wks) in the experimental groups (Fig. 2A). Moreover, Masson’s trichrome staining revealed prominent collagen fibers (liver fibrosis) in the WD and HFC groups (after 16 and 32 wks; Fig. 2A). The METAVIR scoring system was used to evaluate the degree of liver fibrosis. The METAVIR grading criteria were as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with a few septa; F3, portal fibrosis with numerous septa without cirrhosis; F4, cirrhosis and single-blinded diagnosis by a certified pathologist. No significant difference was found among the four groups with respect to fibrosis scores after 16 wks of feeding (Fig. 2B). However, after 32 wks of feeding, the fibrosis scores of the WD and HFC groups were higher than those of the NC and HFD groups; this finding suggests that liver fibrosis was more severe in the WD and HFC groups than in the NC and HFD groups (Fig. 2B). After 16 wks of feeding, no significant changes were noted in the level of collagen deposition in the four groups. However, after 32 wks of feeding, the level of collagen deposition increased significantly in the WD and HFC groups compared with the levels in the NC and HFD groups; this finding indicates severe fibrosis in the WD and HFC groups (Fig. 2C).

In addition to histopathological analysis, this study quantified a clinically used serological biomarker of NAFLD/NASH—hyaluronan. The serum level of hyaluronan was measured through sandwich ELISA (Fig. 2D). No significant changes were noted in the level of hyaluronan in the NC group. Nevertheless, the level of hyaluronan increased significantly in the HFD and WD groups; the highest degree of increase was noted in the HFC group. However, the gradual changes observed in the level of hyaluronan in the HFD group were nonsignificant. These findings indicate prolonged intakes of the WD and HFC increased the level of hyaluronan in the blood of the experimental mice (Fig. 2D). Furthermore, the WD and HFC accelerated NASH progression, leading to advanced, conditions such as liver fibrosis and cirrhosis. In summary, the severity of NAFLD/NASH induced by the three experimental diets could be quantified and scored using clinically relevant diagnostic methods.

**Lipoprotein metabolites are key biomarkers of diet-induced NASH in mice**

The NAFLD/NASH phenotype induced by experimental diets is quantifiable and scalable; thus, the corresponding serological metabolomic characteristics can be aligned. Mouse serum samples were subjected to NMR spectroscopy-based metabolomic profiling followed by bioinformatics analyses. The analytical logic and diagram are presented in Fig. 3. The data corresponding to serological metabolome and collagen deposition (Masson's trichrome staining) were discovered to be correlated. A total of 41 biomarkers and 121 lipoprotein metabolites were assessed (Fig. 3). PCA and heatmap analysis were performed to identify the overall pattern and trends of changes in metabolites. Multiple comparisons were performed to determine the significant differences between the groups (significance was observed after 32 wks of feeding; Kruskal–Walls test) and between the feeding durations (16 and 32 wks; time
effect; Wilcoxon rank-sum test). Additional multiple comparisons were performed for the collagen-related quantitative data (Fig. 3) to determine the differences between the feeding durations; various metabolites were identified in this analysis (Fig. 5A,B). In the case of significant between-group differences in collagen deposition level, correlation and comparative analyses of metabolites and collagen deposition levels were performed. A trimmed list of significant metabolites was obtained (Fig. 6A). Finally, the most significant metabolites (n = 17) were selected from the WD and HFC groups (Fig. 6B).

PCA was performed for the unbiased clustering of the obtained metabolomic data. All metabolites (162 biomarkers), the lipoprotein metabolites alone (121 biomarkers), and the small metabolites alone (41 biomarkers) were assessed. For all metabolites, the first principle component (PC1) accounted for 18.9% of the overall variability, whereas the second principle component (PC2) accounted for 35.3%. The PCA plot based on the top two principal components indicated that all samples could be categorized into NC, 16-wk-experimental-diet, and 32-wk-experimental-diet groups (Supplementary Fig. 1A). For the lipoprotein metabolites, PC1 accounted for 44.1% of the overall variability, whereas PC2 accounted for 22.5%. The PCA plot indicated that all samples could be categorized into NC, 16-wk-experimental-diet, and 32-wk-experimental-diet groups (Fig. 4A). For the small metabolites, PC1 accounted for 18% of the overall variability, whereas PC2 accounted for 12%. The PCA plot indicated that the samples could not be categorized (Fig. 4B). Heatmaps were constructed to visualize the distribution of the significant metabolites across groups. The heatmap corresponding to all metabolites revealed significant differences among the NC, 16-wk-experimental-diet, and 32-wk-experimental-diet groups (Supplementary Fig. 1B). The heatmap corresponding to the lipoprotein metabolites revealed significant differences among the NC, 16-wk-experimental-diet, and 32-wk-experimental-diet groups (Fig. 4C). The heatmaps revealed no significant difference between the groups in terms of serological biomarkers (Fig. 4D). These findings are consistent with the trends of the corresponding PCA results. In summary, the PCA and heatmap analysis indicated that lipoproteins undergo gradual changes and play major roles in the progression of NAFLD/NASH.

VLDL–VLDLR axis is associated with lipid deposition and steatosis symptoms in mouse models of diet-induced NASH

VLDL and LDL are the predominant metabolites in mouse models of diet-induced NASH

The association between phenotypic and metabolomic characteristics indicated that lipoproteins can play a pivotal role in the development of NAFLD/NASH. Differential expression analysis was performed to understand the gradual changes in lipoproteins during the progression of diet-induced NASH. The nonnominal method (Wilcoxon rank-sum test) was used for statistical analysis.[39] Significant biomarkers were selected on the basis of a p value of < 0.05 and an log2FC value of > 1. Pie charts depicting the results of differential gene expression analysis are presented in Fig. 5A. In the HFD group, significant changes were observed in a total of 36 biomarkers, accounting for 14% of all primary biomarkers. VLDL, LDL, and high-density lipoprotein (HDL) accounted for 8%, 58%, and 20%, respectively,
of all biomarkers (Fig. 5A). In the WD group, the primary biomarkers, VLDL, LDL, and HDL accounted for 13%, 15%, 61%, and 11%, respectively, of all biomarkers (Fig. 5A). In the HFC group, the primary biomarkers, VLDL, LDL, and HDL accounted for 14%, 20%, 42%, and 24%, respectively, of all biomarkers (Fig. 5A). The changes in lipoprotein subfractions were ranked and are shown in Fig. 5B. The names, abbreviations, and sizes of all lipoprotein biomarkers are presented in Supplementary Table 3. In the HFD group, the top five metabolites (small LDLs) were L4TG, L4PN, L4AB, L4CH, and L4PL; the expression of these metabolites was considerably upregulated with time. However, the expression of small HDLs, such as H4PL, H4FC, and H4CH, was downregulated. In the WD group, the expression of the following three predominant metabolites was upregulated: the large VLDLs V2TG and V2CH and the large LDL L4TG. The size and number of various lipoproteins exhibited an average distribution; significant increases were particularly observed for LDLs, including for L1TG, L4TG, L2T, L3AB, L3PN, L3CH, L1FC, and L4FC. Regarding HDL, low levels of increases were observed in large HDLs, such as H1CH, H1FC, and H1A1. In the HFC group, considerable changes were noted with time in five VLDLs and two LDLs: the large VLDLs V2TG, V2CH, V1FC, and V4CH and the medium to large LDLs L1TG and L4FC. A significant reduction was noted in one HDL: H4CH. In summary, we observed gradual changes in lipoproteins with the changing severity of NAFLD/NASH. In the HFD, WD, and HFC groups, major changes were observed in small LDLs, large VLDLs and medium LDLs, and large VLDLs and medium to large LDLs, respectively.

To identify the correlation between metabolites and NAFLD/NASH severity, we performed correlation analysis of significant metabolites with collagen deposition (Fig. 6A). A total of 25 significant biomarkers of NAFLD were identified in all groups fed for 32 wks. In total, 34 significant biomarkers of NASH were identified in the WD group; moreover, 60 significant biomarkers of NASH/cirrhosis were identified in the HFC group (Fig. 6A). A comparison of the significant metabolites identified from the experimental groups with the metabolites that were significantly correlated with collagen deposition revealed a high degree of overlapping (Fig. 6B). Pathological analysis (Fig. 2) revealed that although both WD and HFC induced NASH, HFC induced a more severe condition—liver fibrosis (e.g., cirrhosis-like phenotype). Therefore, we further compared the WD group (mild fibrosis) with the HFC group (severe fibrosis) to explore fibrosis-specific metabolites. A total of 17 metabolites were found to be associated with severe liver fibrosis (Fig. 6C). VLDLs and LDLs accounted for 35% and 47%, respectively, of the aforementioned metabolites; both VLDLs and LDLs were found to be predominant in the liver of mice with NASH/cirrhosis (Fig. 6C). The 17 markers included the large VLDLs V1CH, V1PL, V2CH, V2TG, and V4CH; the small LDLs L5PN, L5CH, L5PL, and L5AB; and the HDL HDTG (Fig. 6C). Taken together, the results indicate VLDL and LDL are involved in the development of NAFLD/NASH and may induce cirrhosis. These findings elucidate both the roles of VLDL and LDL as biomarkers of NASH/cirrhosis and the pathophysiological changes that occur during the progression of NASH/cirrhosis.

**Upregulation of VLDLR expression in mouse models of diet-induced NASH**

The liver is the most prominent contributor of lipoproteins because this organ is responsible for both the production and recycling of lipoproteins.[46] Lipoprotein receptors are crucial for systemic lipid
metabolism. The expression of lipoprotein receptors, such as VLDLR, LDL receptor (LDLR), and HDL receptor (SR-B1), in normal organs has been studied in humans and mice.\[^{46, 47}\] VLDL is believed to be produced only by the liver; VLDLR is expressed in the periphery of but not within the liver.\[^{48}\] In our study, the expression of both LDL and VLDL was upregulated in mouse models of NASH/cirrhosis, which prompted us to investigate the receptors of these lipoproteins in diseased liver tissues. The expression and distribution of VLDLR, LDLR, and SR-B1 were evaluated through immunohistochemical analysis (Fig. 7A) and was quantified using ImageJ (Fig. 7B). The expression of VLDLR was not similar between the diet- or feeding time–based groups, with the exception of the HCF group, which was fed for 32 wks (Fig. 7B). After 16 wks of feeding, the expression of LDLR was markedly downregulated in the experimental groups compared with that in the control group; nonetheless, the expression was gradually restored in the WD and HFC groups after 32 wks of feeding (Fig. 7B). Notably, the expression of SR-B1 remained high and did not change with diet (Fig. 7B). In summary, the expression of VLDLR is considerably upregulated in severe liver fibrosis. The findings of increases in the levels of serological VLDL and LDL and the upregulation of VLDLR expression in the severe NASH/cirrhosis of this study indicate a feedforward mechanism for lipid deposition.

**VLDLs serve as the biomarkers of liver fibrosis/cirrhosis in humans**

The expression of large VLDLs and VLDLR are upregulated in mouse models of diet-induced NASH with fibrosis/cirrhosis (Figs. 6C and 7). We analyzed the clinical specimens of a retrospective cohort of patients with liver fibrosis/cirrhosis to identify the correlation between serological metabolites and clinical features. FIB-4 is the most widely used noninvasive tool for evaluating the degree of liver fibrosis. This tool was developed to noninvasively predict severe fibrosis in patients with decompensated liver disease.\[^{49, 50}\] The demographic characteristics of our cohort are summarized in Supplementary Table 4. On the basis of their METAVIR scores, the patients were stratified into mild and severe disease groups. A comparison of the metabolome and differential expression of relevant genes were performed. The results revealed significant increases in the levels of the following metabolites (very large VLDLs) in patients with severe fibrosis: XXL_VLDL_CE, XXL_VLDL_C, L_VLDL_CE, and L_VLDL_C (Fig. 8A). Human and experimental (mouse) NASH disease were similar in terms of the upregulation of the expression of very large VLDLs. In summary, the severity of diet-induced NASH in mouse models can be evaluated to align with clinical diagnostic methods. Metabolomic profiling revealed a likely mechanism of VLDL recycling through VLDLR, which may be involved the pathogenesis of liver fibrosis/cirrhosis.

**Discussion**

In this study, three commonly employed experimental diet–induced NASH models were used to evaluate NASH severity by using clinically relevant diagnostic methods. The serological metabolites associated with NASH/cirrhosis severity were identified. The roles of the lipoprotein–receptor axes in the pathogenesis of diet-induced NASH/cirrhosis in mice were investigated.
Importance of mouse models of diet-induced NASH in clinical diagnosis

NASH is diagnosed on the basis of histopathological features, such as steatosis, hepatocyte ballooning, and lobular inflammation. Although fibrosis is not a histopathological feature of NASH, it can be used to predict the risk of mortality. The METAVIR scoring system is a commonly used tool for diagnosing fibrosis. The pathogenesis and progression of NASH are complex and involve cellular heterogeneity and alterations in the humoral matrix. Histopathological analysis is important in NASH diagnosis. Rodent models of experimental NAFLD/NASH/fibrosis/cirrhosis can be established through diet, genetic modifications, toxin treatment, and a combination of different methods.[11, 15, 51] Few studies have been conducted to systemically compare diet-induced NAFLD/NASH models, evaluate their importance in clinical diagnosis, and identify metabolite biomarkers. The present study was conducted to obtain valuable insight into various decompensated liver diseases, such as NASH, fibrosis, and cirrhosis. Inbred rodents are widely used for studying NAFLD/NASH from a genetic perspective.[52] The present study was conducted using C57BL/6 mice, which are commonly used in transgenic animal studies.

Importance of noninvasive metabolomic tools in NASH diagnosis

The METAVIR scoring system is an invasive tool that involves liver biopsy, which involves the risks of major vein rupture and internal bleeding. Serological biomarkers, such as FIB4, can serve as noninvasive tools for disease diagnosis. However, the precision of clinical diagnosis performed on the basis of FIB4 is low (receiver operating characteristic curve score, approximately 70%).[53, 54] In our study, high-throughput metabolomic profiling was performed with NMR spectroscopy; the results revealed a correlation between the phenotypic and metabolomic characteristics of mouse models of NASH. In addition, a novel biomarker of steatohepatitis was identified.

Regarding the relevance of experimental NASH to human NASH, elevated levels of VLDL, VLDL-cholesterol, and LDL-cholesterol can serve as the biomarkers of the progression of hepatic steatosis to NASH (Fig. 8A). High serum levels of total lipid and cholesterol (VLDL and LDL) are associated with intrahepatic cholesterol accumulation and hepatocyte injury in NASH.[55, 56] The similarity between experimental diet-induced NASH and human NASH in terms of lipoprotein metabolites indicated lipoprotein analysis may be valuable for clinical diagnosis. Therefore, the significance of our study lies in its identification of an association of large VLDLs and LDLs with the progression of NASH in animal models and patients (Fig. 8B).

Most studies conducted using animal models of NASH have explored lipid, glucose, and protein metabolites in the liver. Our findings reveal that the levels of lipoproteins increased with the severity of NASH. Through NMR spectroscopy–based metabolomic profiling, both VLDL and LDL were simultaneously explored in mice and humans. The consistency between the experimental NASH and human NASH in terms of metabolite biomarkers indicated that similarities were present in the
pathophysiological alternations between the mouse and human, and that NMR spectroscopy–based metabolomic profiling can be valuable for research and clinical diagnosis.

**Lipoproteins as the predominant biomarkers of diet-induced NASH in mice**

Serum-insoluble lipids circulate in the bloodstream as lipoproteins, which are macromolecular complexes of free cholesterol, cholesterol esters, triglycerides, phospholipids, and apolipoproteins.[57] The liver is the primary site for the synthesis of LDL (16–30 nm) and VLDL (30–80 nm), which carry lipids and ApoB100 to tissue. An essential lipoprotein for the collection and transportation of extra serum cholesterol is HDL (8–16 nm; ApoA). Structurally, VLDL comprises a triglyceride-enriched core surrounded by a monolayer of phospholipids and incorporated proteins (e.g., ApoB-100), which facilitates the delivery and uptake of VLDL. Notably, most triglycerides incorporated into VLDL are derived from exogenous lipids and not synthesized through de novo lipogenesis.[58, 59] The impaired synthesis of ApoB-100 in patients with NASH may be associated with increased free fatty acid level, disrupted redox balance, hyperinsulinemia, and reduced gene expression, all of which hinder ApoB-100 synthesis and VLDL assembly, thus resulting in intrahepatic lipid accumulation.[60] Additionally, VLDL particles can be converted into LDL particles through hydrolyzation of triglycerides by LPL in the bloodstream.[57]

In patients with NAFLD, the expression of VLDL in the liver is upregulated, leading to increased levels of triglycerides. In addition, the clearance of LDL is reduced, which accelerates the development of atherosclerosis and cardiovascular disease. Excessive lipid storage in the liver promotes the secretion of VLDL and thus dyslipidemia.[61] Furthermore, an increase in the level of oxidized LDL occurs, which induces systemic inflammation.[61]

The increase in the mean size of VLDL in patients with NASH and the reduction in the level of small VLDL in patients with liver fibrosis reflect changes in the number and state of hepatocytes resulting from such diseases.[62] Hepatocytes with increased levels of intracellular lipid can serve as the source of large VLDLs. NASH driven by insulin resistance and an increase in the intrahepatic lipid pool may increase the numbers of large VLDLs and the mean size of VLDLs.[63–65]

**Upregulation of VLDL/VLDLR expression indicates a positive feedforward mechanism for hepatic lipid accumulation**

In adipose tissues, VLDLR is regulated by peroxisome proliferator–activated receptor.[66] Free cholesterol and fatty acid can promote stress response, inflammation, apoptosis, and fibrosis in the liver.[67] Large VLDLs induce the accumulation of triglycerides in macrophages and exhibit a higher affinity for VLDLR binding than do small VLDLs. Disease states may influence VLDL properties. Hepatic secretion of VLDL is impaired in patients with ApoB mutations, which often leads to fatty liver disease because of the excessive intrahepatic accumulation of fat. The extent of steatosis is associated with the size and number of VLDLs in the patient population.[68] Patients with hepatic steatosis and insulin resistance
have increased levels of circulating ApoC-III, which is a strong inhibitor of LPL. After the LPL-mediated hydrolysis of triglycerides, lipoprotein remnants are removed through receptor-mediated pathways, primarily those operated in the liver. Obesity and insulin resistance contribute to reduced LDL clearance by reducing the activities of LDLR and LDLR-related protein 1, among others.[69] The intrahepatic accumulation of LDL due to reduced receptor-mediated uptake may directly inhibit LPL, resulting in a feedforward mechanism that drives lipid deposition and NASH development.[70–72]

The dysregulation of lipid homeostasis in hepatocytes leads to the generation of toxic lipids that impair organelle functions, promoting inflammation, hepatocellular damage, and apoptosis.[70] In patients with NASH, the uptake of circulating lipids, particularly free fatty acids and lipoproteins, by the liver is higher. [73] Because the hepatic secretion of lipoprotein is higher in patients with NAFLD, the deposition of fat in hepatocytes disrupts lipid homeostasis in these cells.[73] Further studies are required to evaluate the quantity and quality of changes in lipid metabolites during the pathogenesis of NASH.

Conclusions

Our findings provide key insight into the pathophysiology and serological metabolomics of experimental diet-induced NASH in relation to human NASH. The finding of an upregulation of lipoprotein expression indicates a feedforward mechanism underlies NASH development, and this mechanism may be targeted for the development of noninvasive diagnostic strategies.

Declarations

Ethics approval and consent to participate: The animal experiments were approved by the Animal Ethics Committee of China Medical University (approval number: CMUIACUC-2021-061). The human study was approved by the Ethics Committee of China Medical University (approval number: CMUH110-REC1-002(CR2) )

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.


Authors’ contributions: Conceptualization, CR Yang and WL Ma; methodology, CR Yang, PC Shen; validation, PY Liao; formal analysis, CR Yang; investigation, YC Hung, S Mehmood, and WC Chang; resources, HS Lai, WC Cheng, and WL Ma; data curation, WC Chang, HC Lai, S Mehmood, and YC Hung;
writing—original draft preparation, CR Yang; writing—review and editing, WL Ma; project administration, WC Cheng; funding acquisition, WC Cheng and WL Ma. All authors read and approved the final manuscript.

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References


**Table**

Table 1 is available in the Supplementary Files section.

**Figures**
Figure 1

Phenotypic characteristics of mouse models of diet-induced NASH. (A) Nutrients and energy source in the three experimental diets. (B) Experimental design for establishing mouse models of nonalcoholic fatty liver disease/NASH. Mice were fed various diets for 16 and 32 wks and then euthanized; liver specimens and blood samples were collected for analysis. (C) Changes in the body weight of mice after 32 wks of feeding. (D) Livers of mice receiving different diets fed for 16 and 32 wks. (E) Liver weight/body weight
ratio of mice fed different diets for 16 wks (left) and 32 wks (right). Statistical significance: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. NC: normal chow; HFD: high-fat diet; WD: Western diet; HFC: high-fat, high-cholesterol diet; NASH, nonalcoholic steatohepatitis.
Evaluation of the severity of diet-induced nonalcoholic steatohepatitis by using clinically relevant diagnostic methods. (A) Analysis of pathological features through hematoxylin–eosin staining. Hepatocyte ballooning and intrahepatic lipid accumulation (droplets) were observed. The severity of liver fibrosis was evaluated through Masson's trichrome staining. The stained samples were observed under a bright-field microscope; scale bar = 200 mm. (B) METAVIR scores were quantified by a licensed pathologist who was blinded to the group allocation. (C) Masson's trichrome staining scores for the observed collagen fibers were quantified using ImageJ. (D) Serum levels of hyaluronan were measured through enzyme-linked immunosorbent assay. Statistical significance: *p < 0.05, ***p < 0.001, and ****p < 0.0001, compared with normal chow; #p < 0.05, ###p < 0.001, and ####p < 0.0001, compared with the high-fat diet; $p < 0.05 and $$p < 0.01, compared with the Western diet.
Figure 3

Strategies for metabolomic analysis.

Metabolomic profiles (light green) and collagen fiber quantitation data (yellow) were analyzed through differential gene expression and correlation analyses. The correlation of serological metabolites with fibrosis severity was investigated (dark green), and the list of significant metabolites was shortened.
Unbiased clustering was performed through primary component analysis and heatmap analysis; the results are presented in Figure 4A,B. The results of a comparison of the 16- and 32-wk feeding durations in terms of metabolomic changes in the experimental mice (time effect) are presented in Fig. 5A,B (Wilcoxon rank-sum test). A multigroup comparison (Kruskal–Wallis test) was performed to differentiate between the significant metabolites. Significant metabolites were compared with significant collagen deposition (Kruskal–Wallis test; yellow squares); the correlation between collagen deposition and metabolome was investigated (correlation analysis; yellow square; Fig. 6A). The intersection of significant metabolites among the 32-wk, WD, and HFC groups is depicted in Figure 6B (dark-colored square), and the shortened metabolite list is presented in Figure 6C.
Lipoproteins as predominant metabolites in mouse models of diet-induced nonalcoholic steatohepatitis.

(A) Plot generated through principal component analysis (PCA) of the lipoproteins of mice fed with NC, HFD, WD, or HFC (16 and 32 wks). (B) Plot generated through the PCA of the serological metabolites of mice fed with NC, HFD, WD, or HFC (16 and 32 wks). PC1: principal component 1; PC2: principal component 2.
component 2. Each point represents the metabolite profile of a biological replicate. (C) Heatmap exhibiting prominent differences in lipoprotein patterns among NC, 16-wk experimental diet, and 32-wk experimental diet. (D) Heatmap exhibiting no distinct pattern of serological metabolites. NC: normal chow; HFD: high-fat diet; WD: Western diet; and HFC: high-fat, high-cholesterol diet.

Figure 5
Changes in lipoprotein metabolites with progression of diet-induced nonalcoholic steatohepatitis.

(A) Pie chart presenting numbers and proportions of lipoprotein metabolites exhibiting gradual changes with diet. (B) Differential expression (32 vs. 16 wks) of lipoprotein metabolites exhibiting gradual changes with diet. Significant lipoproteins were selected on the basis of a $p$ value of $<0.05$ and a log2FC value of $>1$. Green part represents very low-density lipoprotein, yellow part represents low-density lipoprotein, and grey part represents high-density lipoprotein.
Expression levels of very low-density lipoprotein and low-density lipoprotein are strongly correlated with the severity of nonalcoholic steatohepatitis/fibrosis. (A) Correlation between serological metabolites and collagen scores. Significant lipoproteins were selected on the basis of a $p$ value of $<0.05$ and a correlation coefficient of $>0.3$. (B) Integrated analysis of significant metabolites and metabolites strongly associated with fibrosis. Blue indicates significant metabolites, whereas yellow indicates metabolites strongly correlated with fibrosis. (C) Shortened list of 17 significant metabolites identified from the comparison between HFC and WD groups (Table on the left). The pie chart presents the proportions of the 17 significant metabolites.
Figure 7

Expression levels of lipoprotein receptors in mouse models of diet-induced NASH. (A) Results of immunohistochemical staining performed to measure the expression levels of very low-density lipoprotein receptor, low-density lipoprotein receptor, and high-density lipoprotein receptor (SR-B1) in the livers of mice with diet-induced NASH. The brown indicates receptor expression. (B) Receptor expression levels were quantified using ImageJ and analyzed using GraphPad Prism (version 8). The white, black,
blue, and red dots indicate NC, HFD, WD, and HFC, respectively. Statistical significance: \(*p < 0.05\), compared with NC. NC: normal chow; HFC: high-fat diet; WD: Western diet; HFC: high-fat, high-cholesterol; and NASH: nonalcoholic steatohepatitis.

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Very low–density lipoprotein and low-density lipoprotein as key biomarkers of fibrosis/cirrhosis in humans. (A) Significant metabolites identified through the differential gene expression analysis of a retrospective cohort of patients with fibrosis/cirrhosis. Log2 fold changes were calculated by comparing the FIB4 scores of patients with advanced disease (F3 or F4) with those of patients with no or mild fibrosis (F0–F2). Significant metabolites associated with cirrhosis. Log2 fold changes were determined by comparing patients with cirrhosis with those without cirrhosis. (B) Changes in lipoproteins during the progression of nonalcoholic steatohepatitis/fibrosis/cirrhosis in mice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- 230925supplementaltext.pdf