



Original Article



Intestinal Depletion of TM6SF2 Exacerbates High-fat Diet-induced Metabolic Dysfunction-associated Steatotic Liver Disease through the Gut-liver Axis

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Abstract

Background and Aims: Metabolic dysfunction-associated steatotic liver disease (MASLD), is the most common form of chronic liver disease worldwide. This study aimed to explore the role of TM6SF2 in high-fat diet (HFD)-induced MASLD through the gut-liver axis. **Methods:** The TM6SF2 gut-specific knockout (TM6SF2 GKO) mouse was constructed using CRISPR/Cas9 technology. TM6SF2 GKO and wild-type (CON) mice were fed either a HFD or a control diet for 16 weeks to induce MASLD. Blood, liver, and intestinal lipid content, as well as gut microbiota and serum metabolites, were then analyzed. **Results:** TM6SF2 GKO mice fed an HFD showed elevated liver and intestinal lipid deposition compared to CON mice. The gut microbiota of HFD-fed TM6SF2 GKO mice exhibited a decreased Firmicutes/Bacteroidetes ratio compared to HFD-fed CON mice. The HFD also reduced the diversity and abundance of the microbiota and altered its composition. Aspartate aminotransferase, alanine aminotransferase, and total cholesterol levels were higher in HFD-fed TM6SF2 GKO mice compared to CON mice, while triglyceride levels were lower. Serum metabolite analysis revealed that HFD-fed TM6SF2 GKO mice had an increase in the expression of 17 metabolites (e.g., LPC [18:0/0-0]) and a decrease in 22 metabolites (e.g., benzene sulfate). The differential metabolites of LPC (18:0/0-0) may serve as HFD-fed TM6SF2 serum biomarkers, leading to MASLD exacerbation in GKO mice. **Conclusions:** TM6SF2 GKO aggravates liver lipid accumulation and liver injury in MASLD mice. TM6SF2 may play an important role in regulating intestinal flora and the progression of MASLD through the gut-liver axis.

Keywords: Transmembrane 6 superfamily member 2; Metabolic dysfunction-associated steatotic liver disease; High-fat diet; Lipid metabolism; Gut microbiota; Gut-liver axis.

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Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD), previously known as nonalcoholic fatty liver disease (NAFLD), is the most common form of chronic liver disease worldwide. It is considered a complex condition with a spectrum of histopathological changes that range from steatosis to metabolic dysfunction-associated steatohepatitis, fibrosis, and cirrhosis.¹ The pathogenesis of MASLD contains various factors, including uncontrolled inflammation, lipotoxicity, oxidative stress, and endoplasmic reticulum stress.² In addition, accumulating investigations have implicated the role of gut microbiota dysbiosis in the development and progression of MASLD.^{3,4} Alterations in the composition and function of the gut microbiota, as well as microbial metabolism, have been recognized as additional cofactors that may accelerate the development of MASLD.⁵

Transmembrane 6 superfamily member 2 (TM6SF2) is a transmembrane protein mainly expressed in the intestines and liver.⁶ Its E167K variant (rs58542926) has been shown to be associated with MASLD.⁷ Under high-fat diet (HFD) conditions, TM6SF2 reduces liver lipid accumulation and protects the liver. Conversely, the absence of TM6SF2 exacerbates liver lipid accumulation.⁸ It is thought that this variant increases the risk of liver fat accumulation by promoting lipid retention and impairing very low-density lipoprotein (VLDL) export.⁹ Furthermore, hepatology guidelines from the European Association for the Study of the Liver confirm that carriers of this variant have higher liver fat content and an increased risk of nonalcoholic steatohepatitis (NASH).¹⁰

Although TM6SF2 plays an essential role in lipid metabolism, MASLD is a multifactorial disease. The interaction between diet and the microbiota can lead to changes in

microbiota composition, resulting in impaired intestinal permeability. Meanwhile, altered microbiota may release harmful bacterial by-products that reach the liver through the portal vein, leading to intrahepatic metabolic disorders and liver inflammation.¹¹

Research has begun to reveal the relationship between host genetics, gut microbiota, and environmental factors, such as lifestyle, in MASLD. While most studies have focused on determining how gut microbiome-produced metabolites affect host genes, increasing evidence suggests that the host genotype can influence the diversity and abundance of microbial communities. Ussar *et al.* conducted an investigation of metabolic parameters and microbiota across mice with different susceptibilities to obesity and diabetes, which were housed in a common environment. They found strong interactions between the microbiota, diet, breeding conditions, and metabolic phenotypes. These findings demonstrate the complex cross-talk among the host genetic background, gut microbiota, and diet.¹² However, the current lack of effective interventions for MASLD indicates the need for a deeper understanding of these interactions.

In the present study, the TM6SF2 gut-specific knockout (TM6SF2 GKO) mouse model was constructed to explore the effects of TM6SF2 GKO on body weight, liver index, hepatitis index, liver lipid index, and intestinal lipid index. In addition, the role of TM6SF2 GKO in intestinal flora diversity/composition and the serum metabolomic profile in MASLD *in vivo* was explored. These findings may contribute to the characterization of MASLD diagnostic/prognostic biomarkers and the identification of potential therapeutic drugs for MASLD.

Methods

Experimental animals

Cre-locus of X-over P1 (LoxP) sites were inserted into flank exons of the murine *TM6SF2* gene, and the inserts were verified by sequencing. The pups with transmission of the heterozygous *TM6SF2*^{fl/+} were confirmed by polymerase chain reaction (PCR) analysis. Mice carrying the *TM6SF2*^{fl/+} allele were bred until a *TM6SF2*^{fl/fl} mice colony was generated. Subsequently, *TM6SF2*^{fl/fl} mice were mated with mice that harbored intestinal-specific Cre recombinase under the control of the villin promoter (Vil-Cre, Nanjing University-Nanjing Institute of Biomedicine) to produce both flox control and *TM6SF2* GKO mice. The pups were genotyped by PCR using the following primers: LoxP_Foward (F) 5'-CAGTGTCAGATTAGTTGGC-3', LoxP_Reverse (R): 5'-CACCGAAGTTATACAGTCTGAT-3'; Vil-Cre_F: 5'-GTGTTTGGTTTGGTTTCTCTGCATAAGA-3', Vil-Cre_R: 5'-GCAGGCAAATTTGGTGTACGGTCA-3'.

Next, mice were divided into four groups based on genotype and treatment ($n = 8$): *TM6SF2* GKO-HFD (*TM6SF2* GKO mice fed with HFD), CON-HFD (common mice fed with HFD), *TM6SF2* GKO-CD (*TM6SF2* GKO mice fed with control diet [CD]), and CON-CD (common mice fed with CD) groups. The HFD (60% fat, 20% protein, 20% carbohydrate, 5.21 kcal/g) was purchased from Research Diets, USA, and the CD (10% fat, 20% protein, 70% carbohydrate, 3.85 kcal/g) was purchased from Jiangsu Synergy Pharmaceutical BioEngineering Co., Ltd. The diets for all mice were maintained for 16 weeks.

Quantitative real-time PCR analysis

Total RNA was extracted from tissues using RNAiso Plus (#9108, Takara), and complementary DNA was generated using the PrimeScrip™ RT Reagent Kit (RR037B, Takara), according to the manufacturer's instructions. The genes of interest were amplified in triplicate using the QuantiNova™ SYBR

Green PCR Kit (#208052, Qiagen). The expression levels of the *TM6SF2* gene were normalized to β -actin mRNA. The primers used were: *TM6SF2*_F: 5'-CGGACTGGGCTTGGTATTT-3', *TM6SF2*_R: 5'-CTTGGTCCTGTGGCGAAGAT-3'; β -actin_F: 5'-CAGCTTCTTTCAGCTCCTT-3', β -actin_R: 5'-CACGATGGAGGGAATACAG-3'.

Western blot analysis

Tissues were solubilized in M-PER mammalian protein extraction reagent supplemented with protease inhibitors. After determining the protein concentration, equal amounts of protein were separated by 6–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were then incubated in 5% skim milk in Tris-buffered saline with Tween 20 for one hour at room temperature, followed by overnight incubation with primary antibodies against *TM6SF2* (1:2,000; TA335280, Origene) or β -actin (1:5,000; MA1-140, Invitrogen) at 4°C. After washing with Tris-buffered saline with Tween 20, the membranes were incubated with a secondary antibody (goat anti-rabbit IgG, 1:5,000; Jackson ImmunoResearch) for one hour at room temperature. Membranes were developed using enhanced chemiluminescence reagents (Amersham Biosciences), and the proteins were visualized with the ChemiDoc XRS+ imager (Bio-Rad, USA). Finally, the relative expression level of *TM6SF2* was normalized to β -actin and quantified using the Image Lab software (Bio-Rad, USA).

Biochemical analysis of blood and tissue samples

After a 6-h fasting period, blood samples were collected from mice, and serum was obtained by centrifugation (3,000 g, 10 min). The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides (TG), total cholesterol (TC), and low-density lipoprotein (LDL) were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer's instructions. The following specific kits were used: ALT assay kit (C009-3-1), AST assay kit (C010-3-1), TG assay kit (A111-2-1), TC assay kit (A110-2-1), HDL assay kit (A112-2-1), and LDL assay kit (A113-2-1).

To detect the levels of TG and TC in liver and intestinal tissues, commercially available kits from Beijing Pully Co., Ltd. were used (TG assay kit: E1025-105, TC assay kit: E1026-105).

Gut microbiota detection

Genomic DNA was extracted from mouse feces samples using the E.Z.N.A. Soil DNA kit (Omega Bio-tek, Inc., USA). The V3-4 hypervariable region of the bacterial 16S rRNA gene was amplified using the following universal primers: 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). For each sample, an eight-digit barcode sequence was added to the 5' end of both forward and reverse primers (provided by Allwegene Company, Beijing).

Deep sequencing was performed using the Illumina MiSeq/Novaseq platform (Illumina, Inc., USA) at Beijing Allwegene Technology Co., Ltd. Sequencing data processing was performed using the Illumina Analysis Pipeline Version 2.6 (Illumina, Inc., USA). Bioinformatics analysis was partially performed with the assistance of Beijing Ovison Gene Technology Co., Ltd.

Detection of serum metabolites in mice

Serum samples (100 μ L) were mixed with 200 μ L of extract solution (acetonitrile: methanol = 1:1, v/v) for metabolite extraction. The mixture was vortexed (30 s, room tempera-

ture), sonicated (10 m, 0°C), and incubated at -40°C for one hour. After centrifugation (12,000 rpm, 15 m, 4°C), the supernatant was transferred to a clean glass vial for liquid chromatography/mass spectrometry analysis. Metabolite profiling was conducted using the Vanquish ultra-high performance liquid chromatography system (ThermoFisher Scientific) coupled with the Orbitrap Exploris 120 mass spectrometer (ThermoFisher Scientific). Part of the experiment and data analysis were performed with the assistance of Beijing Ovison Gene Technology Co., Ltd.

Statistical methods

Data analysis and graphics generation were performed using GraphPad Prism 9 software (GraphPad, USA). All experimental data are presented as mean $\pm$ standard error of the mean (mean $\pm$ SEM). For comparisons between the two groups, an independent sample *t*-test was used. For comparisons among multiple groups, the one-way analysis of variance was applied. A *p*-value of <0.05 was considered statistically significant. Asterisk symbols were used to indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

Establishment and characterization of TM6SF2 GKO mice

To determine the tissue distribution of TM6SF2, its expression was assessed in various mouse tissues using quantitative real-time PCR. The results revealed that TM6SF2 is abundantly expressed in the intestine and liver, but barely detectable in other examined tissues (Fig. 1A). Based on these observations, the TM6SF2 GKO mouse model was established to investigate the role of intestinal TM6SF2 in MASLD pathogenesis.

The intestine-specific depletion of the *TM6SF2* gene was confirmed by PCR analysis (Fig. 1B). To validate the knockout efficiency, TM6SF2 mRNA (Fig. 1C) and protein (Fig. 1D) levels in the small intestine of CON and TM6SF2 GKO mice were measured. Additionally, TM6SF2 expression was assessed in the liver of CON and TM6SF2 GKO mice, and it was found that *Vil-Cre* did not induce a reduction in TM6SF2 in the liver (Fig. 1D). Importantly, TM6SF2 expression was significantly decreased in the small intestine of GKO mice, indicating the successful establishment of the TM6SF2 GKO mouse strain.

During the 16-week modeling period, the body weights of all mice were recorded. The results revealed that HFD-fed mice gained more weight compared to their CD-fed littermates (Fig. 1E). Furthermore, the livers obtained from mice in the TM6SF2 GKO-HFD group exhibited a mottled appearance and light-yellow color compared to the other groups, suggesting lipid accumulation in the liver (Fig. 1F). Liver index analysis showed that compared to CON mice, TM6SF2 GKO mice had enlarged livers under both HFD and CD conditions (Fig. 1G). Overall, the data suggest that TM6SF2 plays an important role in lipid metabolism.

Effect of TM6SF2 on hepatic and intestinal lipid metabolism

Next, the effect of TM6SF2 on lipid metabolism in mice was examined by determining the levels of TG, TC, and LDL in the liver and intestines. Liver TG content was significantly elevated in TM6SF2 GKO mice fed with HFD compared to HFD-fed controls, while no significant difference was observed under CD conditions. Additionally, liver TC content was significantly lower in mice from the TM6SF2 GKO-CD group compared to the CON-CD group, while no significant difference was observed in HFD-fed mice. Liver LDL levels were comparable

between CON and TM6SF2 GKO mice under both HFD and CD conditions (Fig. 2A). Oil Red O staining revealed an increase in lipid content in the liver of HFD-fed mice. Among these, TM6SF2 GKO mice had the highest lipid deposition, as reflected by large granular lipid droplets in hepatocytes (Fig. 2B). Furthermore, hematoxylin and eosin staining suggested that HFD-induced hepatic steatosis was more severe in TM6SF2 GKO mice (Fig. 2C).

The content of TG, TC, and LDL in the intestines was also evaluated. TG content in the intestines was significantly higher in HFD-fed TM6SF2 GKO mice compared to HFD-fed CON mice. A similar trend was observed in CD-fed mice, although the difference did not reach statistical significance. However, no significant differences in intestinal TC and LDL content were observed between the two groups (Fig. 2D). Both Oil Red O staining and H&E staining revealed an increase in lipid deposition in the small intestines of HFD-fed mice compared to their CD-fed littermates. Among the HFD-fed groups, TM6SF2 GKO mice had higher levels of lipid accumulation (Fig. 2E and F). Additionally, Alcian Blue staining of small intestinal tissues revealed that the number of goblet cells was lower in HFD-fed mice compared to CD-fed mice, and intestinal permeability was increased (Fig. 2G).

Effects of TM6SF2 on gut microbiota composition and diversity

Next, 16S rRNA sequencing was conducted to analyze the intestinal microbiota composition in the experimental groups. Shannon-Wiener curves revealed that as the number of reads per sample increased, the curve reached a plateau, indicating that the sequencing depth was sufficient to capture most of the microbiomes in the fecal samples (Fig. 3A). Alpha diversity analysis suggested that the HFD condition reduced species richness of the microbiome compared to the CD condition. Notably, TM6SF2 GKO mice on HFD exhibited the lowest diversity among all groups (Fig. 3B).

Partial least squares discriminant analysis based on operational taxonomic units was performed to maximize the differences between the sample groups (Fig. 3C). The clear separation between the HFD and CD groups indicated that diet was the major factor affecting microbiota composition. Meanwhile, the separation between the TM6SF2 GKO and CON groups suggested that TM6SF2 depletion dramatically influenced gut microbiota composition.

Taxonomic analysis of the gut microbiota composition was conducted at multiple levels (from phylum to species). The Firmicutes/Bacteroidetes ratio was found to increase in HFD-fed mice compared to CON mice, but this ratio decreased in HFD-fed TM6SF2 GKO mice compared to HFD-fed CON mice (Fig. 4A–F). Furthermore, there was an increase in the abundance of *Desulfovibrio*, *Clostridia_UCG_014*, and *CandidatusArthromitus*, while the abundance of *Intestinimonas* and *Ruminococcustorques* group decreased in the TM6SF2 GKO-HFD group (Fig. 5A). Meanwhile, the abundance of *Lachnospiraceae_UCG_006*, and *Rikenellia* increased, while the abundance of *Proteobacteria*, *Christensenellales*, and *Clostridiales* decreased in CD-fed TM6SF2 GKO mice (Fig. 5B).

Effect of TM6SF2 on liver function

To understand the role of TM6SF2 in lipid metabolism and liver function, serum levels of AST, ALT, TC, TG, LDL, and HDL were measured. HFD-fed TM6SF2 GKO mice had significantly higher levels of AST, ALT, TC, and HDL compared to HFD-fed CON mice (Fig. 6A, B, D, and F). This suggests that TM6SF2 deficiency exaggerates HFD-induced alterations in liver function and lipid metabolism. Furthermore, the differences in

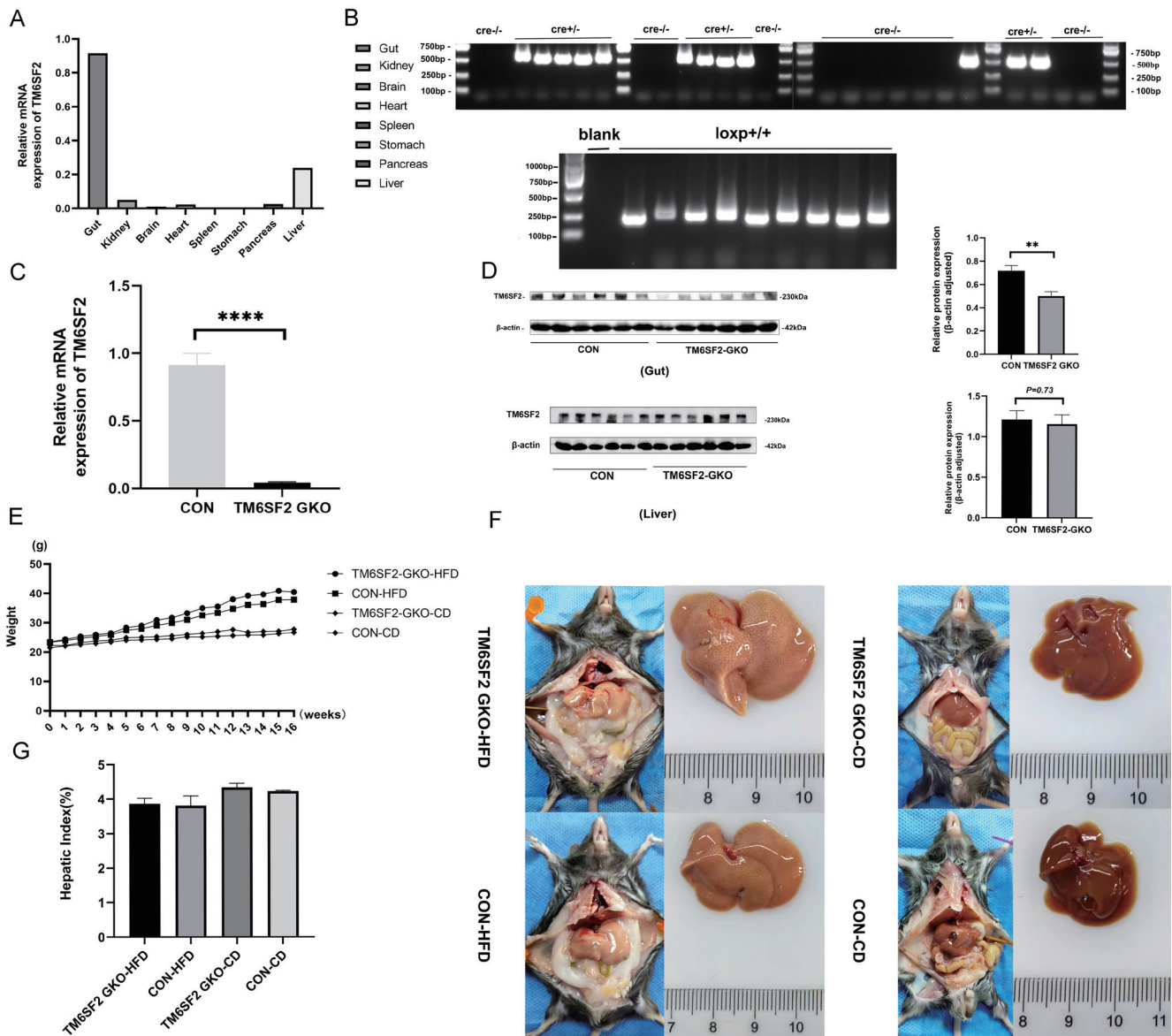


Fig. 1. Establishment and general effects of TM6SF2 gut-specific knockout. (A) Relative mRNA expression of TM6SF2 in different tissues; (B) Gene expression of TM6SF2 in mouse intestines at the DNA level; (C) Gene expression of TM6SF2 at the mRNA level; (D) Gene expression of TM6SF2 at the protein level; (E) Sixteen-week body weight change curve for the four groups of mice; (F) Anatomy images for the four groups of mice after sixteen-week weeks of modeling; (G) Liver index analysis for the four groups of mice after sixteen-week weeks of modeling. ** $p < 0.01$, **** $p < 0.0001$.

serum levels of AST, ALT, and TC between the two groups under CD conditions were not significant (Fig. 6A, B, and D). Additionally, no significant differences in serum TG and LDL levels were observed between TM6SF2 GKO and CON mice under HFD conditions (Fig. 6C and E).

Sequencing data confirmed that TM6SF2 GKO and HFD altered the gut microbiome composition in mice. These changes in serum biomarkers may be associated with these microbiome alterations. Thus, it is reasonable to speculate that TM6SF2 depletion and HFD synergistically altered the gut microbiota, resulting in changes in lipid metabolism and liver function.

Effect of gut-specific knockout of TM6SF2 on serum metabolites in mice

Ultra High-Performance Liquid Chromatography-Mass Spec-

trometry (hereinafter referred to asUHPLC-QE-MS) analysis was conducted to identify the serum metabolite profiles in CON and TM6SF2 GKO mice under HFD conditions (Fig. 7A–C). In HFD-fed TM6SF2 GKO mice, 17 upregulated metabolites were identified: LPC (18:0/0:0), 1-O-Hexadecyl-2-O-acetyl-sn-glycerol-3-phosphorylcholine, 2-1-Octadecyl-acetyl-sn-glycerol-3-phosphocholine, deoxyguanosine triphosphate, adenosine 5'-triphosphate, AKBA, oleoylcarnitine, M433T120, lycium-D, ethyl glucuronide, 2-Keto-butyric acid, hexenoylcarnitine, arborinine, antibiotic TAN 1446A, SM (d34:1), megestrolacetate, and M262T130. Conversely, 22 downregulated metabolites were identified in HFD-fed TM6SF2 GKO mice: phenylsulfate, indolepropionic acid, indole-3-methyl acetate, 13,14-dihydro-16,16-difluoroprostaglandin, J2,5-[3-[(1S,2S,4R)-bicyclo[2.2.1]

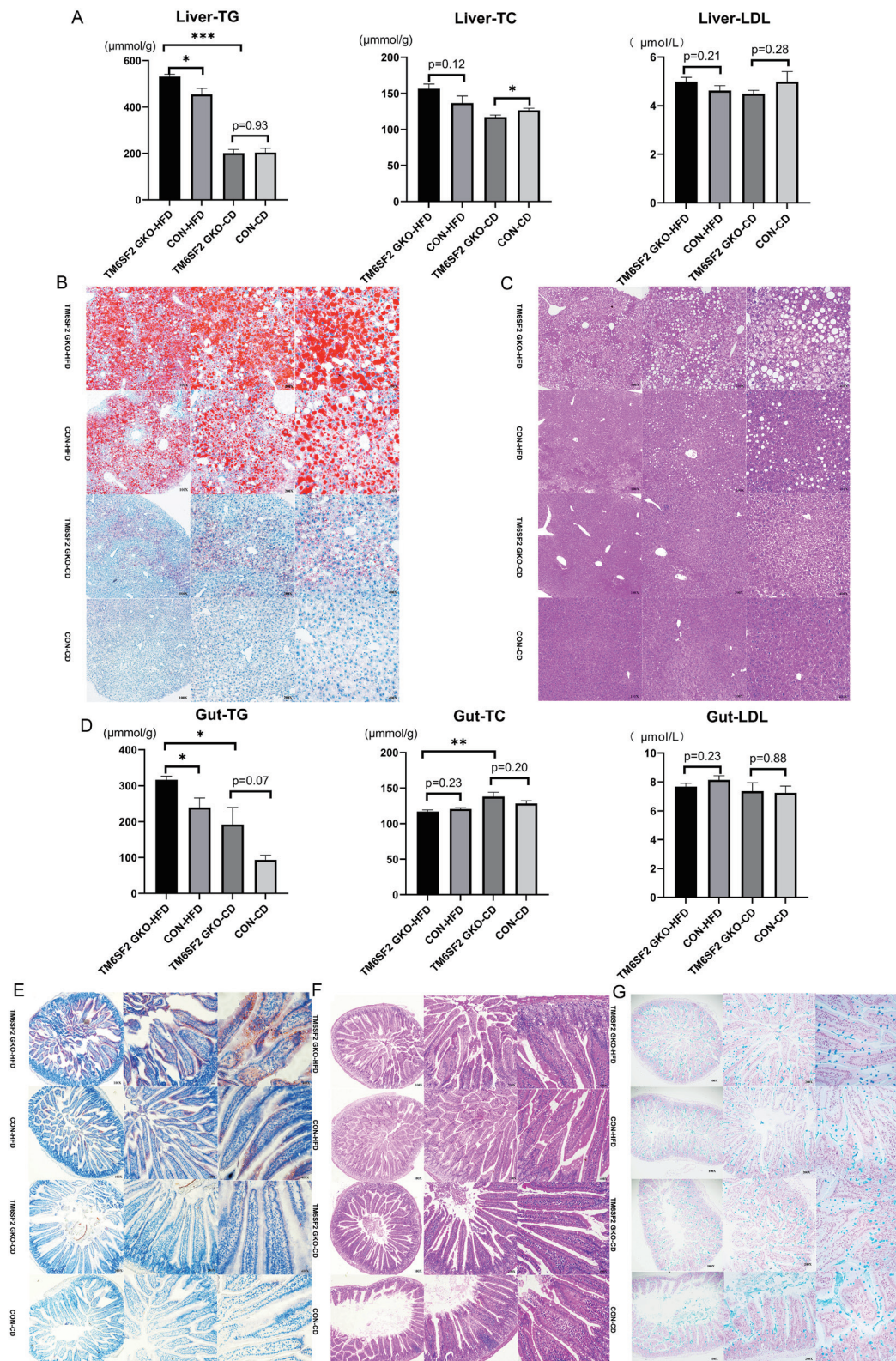


Fig. 2. Effects of TM6SF2 gut-specific knockout on the liver and gut. (A) Liver TG, TC, and LDL content analysis for the four groups of mice; (B) Oil Red O staining of the liver for the four groups of mice; (C) Liver H&E staining for the four groups of mice; (D) Small intestine TG, TC, and LDL content analysis for the four groups of mice; (E) Oil Red O staining of the small intestine for the four groups of mice; (F) H&E staining of the small intestine for the four groups of mice; (G) Alcian Blue staining for the four groups of mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein; H&E, hematoxylin and eosin stain.

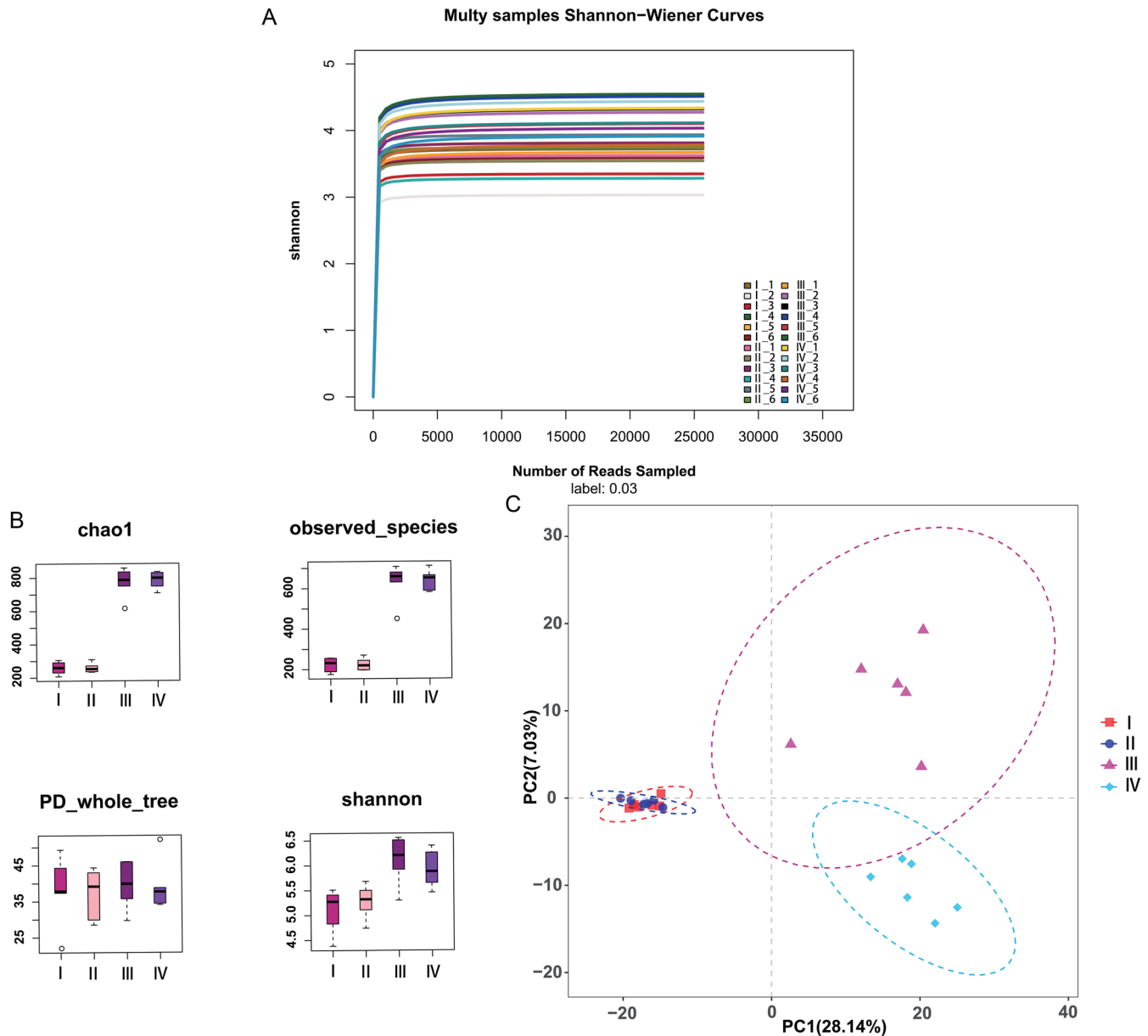


Fig. 3. Analysis of gut microbiota in mouse fecal samples. (A) Shannon-Wiener curve of accuracy for the fecal microbiota detection data; (B) Box plot for the alpha diversity index of the fecal microbiota detection data of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (C) Partial least squares discriminant analysis based on the OTUs of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice. I, TM6SF2 GKO-HFD; II, CON-HFD; III, TM6SF2 GKO-CD; IV, CON-CD.

hept-2-yloxy]-4-methoxyphenyl]tetrahydro-2(1H)-pyrimidinone, SM (d18:1/22:0), 1-Palmitoyl-2-thiopalmityl phosphatidylcholine, SM (d18:1/20:0), D-alpha-tocopherol succinate, SM (d16:1/24:1[15Z]), pongachin, 1-(p-Tolyl) cyclopropanecarboxylic acid, tetrapropylammonium cation, PC (34:2), 5-(3-Phenylpropyl)-1H-1,2,3,4-tetrazole, (Z)-N-Feruloyl-5-hydroxyanthranilic acid, PE (18:3[9Z,12Z,15Z]/P-18:1[9Z]), 5-azacytosine, SM (d17:1/24:1[15Z]), alpha-truxillic acid, 3,3-diaminobenzidine, and Pro-Asp (Fig. 7D-F). These altered metabolites suggest that TM6SF2 plays a critical role in lipid metabolism under HFD conditions.

The KEGG pathway analysis of the differential metabolites revealed that mice in the TM6SF2 GKO-HFD group exhibited a downregulated sphingolipid signaling pathway, specifically

involving SM (d18:1/20:0). In addition, an upregulation was observed in choline metabolism related to cancer and erythrocytosis, which involved LPC (18:0/0:0) (Fig. 8A-C). These findings suggest that TM6SF2 may participate in the regulation of lipid and metabolic signaling pathways under HFD conditions.

Correlation analysis revealed significant associations between LPC (18:0/0:0) and arborinine, 1-O-Hexadecyl-2-O-acetyl-sn-glycerol-3-phosphorylcholine, antibiotic TAN1446A, and oleoylcarnitine, while oleoylcarnitine showed a notable correlation with 2-ketobutyric acid (Fig. 8D). These observations further indicate a connection between TM6SF2 and metabolic signaling pathways.

Another correlation analysis was performed to determine

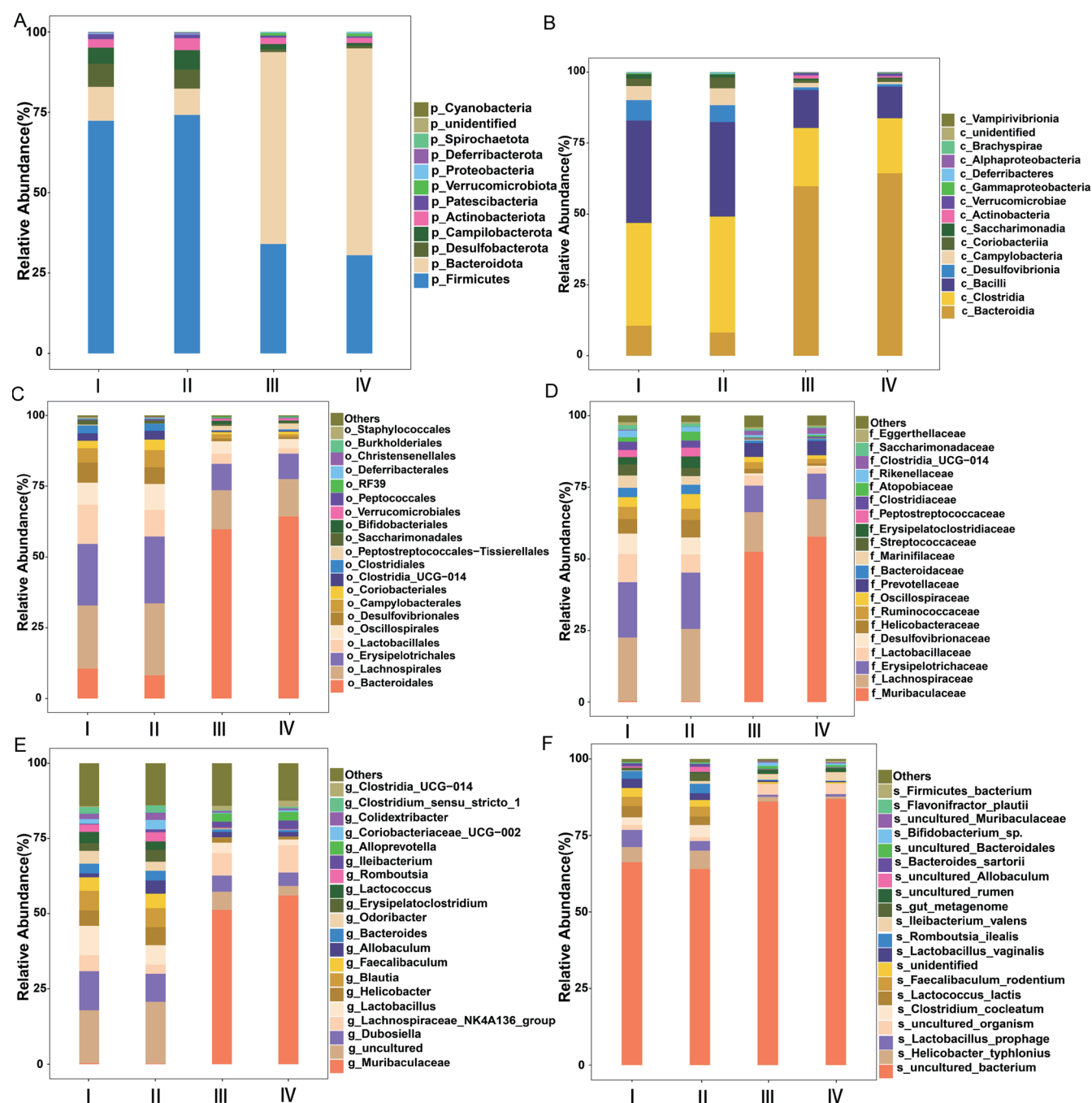


Fig. 4. Bar graph illustrating the analysis of species composition in the four mice populations. Difference in species (A, phylum level; B, class level; C, order level; D, family level; E, genus level; F, species level) in the Wilcoxon test for the fecal microbiota detection data of control diet fed TM6SF2 gut-specific knockout and wild-type mice. I, TM6SF2 GKO-HFD; II, CON-HFD; III, TM6SF2 GKO-CD; IV, CON-CD.

the impact of TM6SF2 on gut microbiome alterations and serum metabolites under HFD conditions. The findings revealed associations with *Desulfovibrio* and *Clostridia_UCG_0* (Fig. 9A). Furthermore, receiver operating characteristic (ROC) curve analysis was performed to determine the potential of differentially expressed metabolites as biomarkers. It was found that several metabolites had high area under the curve values, such as SM (d18:1/20:0):0.75, LPC (18:0/0:0):1, arborinine: 1, antibiotic TAN 1446A: 0.917, oleoylcarnitine:

0.944, and 2-ketobutyric acid: 0.917 (Fig. 9B). These results demonstrate that these metabolites may be used as potential diagnostic biomarkers for TM6SF2-associated HFD-induced MASLD.

Discussion

MASLD is recognized as a complex disease associated with the dysregulation of multiple interrelated biological path-

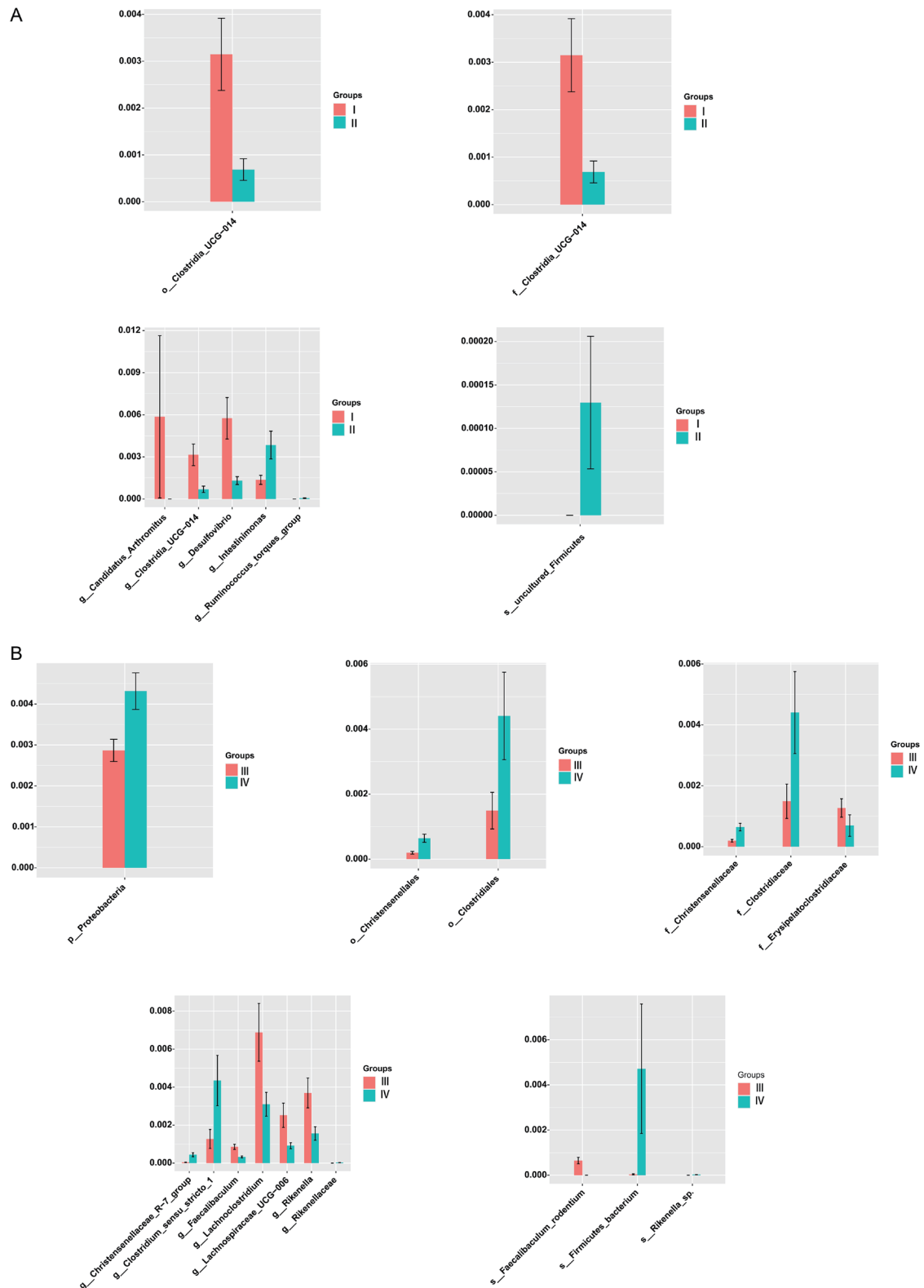


Fig. 5. Effects of TM6SF2 gut-specific knockout on the gut microbiota in mice. (A) Difference in species in the Wilcoxon test for the fecal microbiota detection data of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice (order level, family level, genus level, and species level); (B) Difference in species in the Wilcoxon test for the fecal microbiota detection data of control diet fed TM6SF2 gut-specific knockout and wild-type mice (phylum level, order level, family level, genus level, and species level).

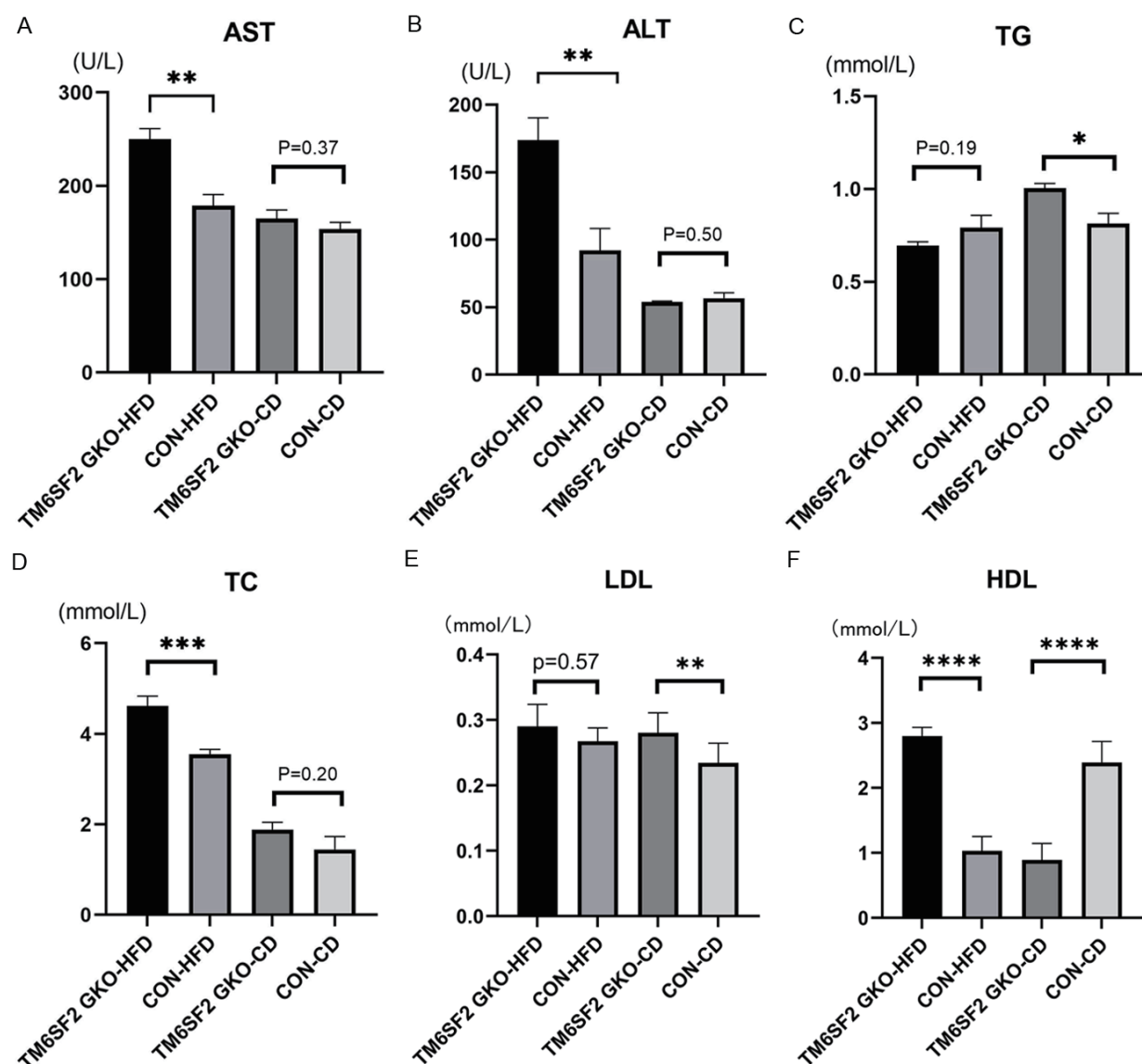


Fig. 6. Effect of TM6SF2 gut-specific knockout on serum inflammation and lipid indexes in mice. (A) Serum level of AST analysis for the four groups of mice; (B) Serum level of ALT analysis for the four groups of mice; (C) Serum level of TG analysis for the four groups of mice; (D) Serum level of TC analysis for the four groups of mice; (E) Serum level of LDL analysis for the four groups of mice; (F) Serum level of HDL analysis for the four groups of mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

ways.¹³ Previous studies have revealed that the TM6SF2 E167K variant (rs58542926) plays critical roles in liver fat metabolism.¹⁴ Furthermore, studies have suggested that this variant leads to liver TG accumulation and MASLD.¹³ However, the mechanism by which this variant contributes to MASLD remains unknown. The present study used a gut-specific knockout model under HFD to provide novel insights into the role of TM6SF2 in MASLD pathogenesis.

The liver and intestines are the two main sources of lipids in the body. The gut absorbs dietary lipids and releases them in the form of chylomicrons, while the liver synthesizes lipids and secretes them as VLDLs.^{15,16} TM6SF2 is mainly expressed in the small intestines and liver, raising the possibility that TM6SF2 regulates lipid metabolism in these organs. Previous studies on liver- or gut-specific TM6SF2 knockout

mice revealed that the depletion of TM6SF2 induces an increase in TG accumulation in TM6SF2-depleted organs.^{17,18} In addition, the inactivation of TM6SF2 in zebrafish induced TG accumulation in the intestine.¹⁹ The present study revealed that although the HFD alone induced significant fat accumulation in the liver, TM6SF2 depletion appeared to exacerbate the HFD-induced fat accumulation. Interestingly, these results suggest that TM6SF2 deficiency in the intestines may play a similar role in the accumulated liver TG content in the TM6SF2^{E167K} knock-in mouse model, as shown in our team's recent research.²⁰ These findings suggest that TM6SF2 deficiency may increase the vulnerability of the host to diet-induced liver damage. The present findings on accumulated liver and intestinal TG content, combined with the reduction in circulating TG levels, are consistent with reports

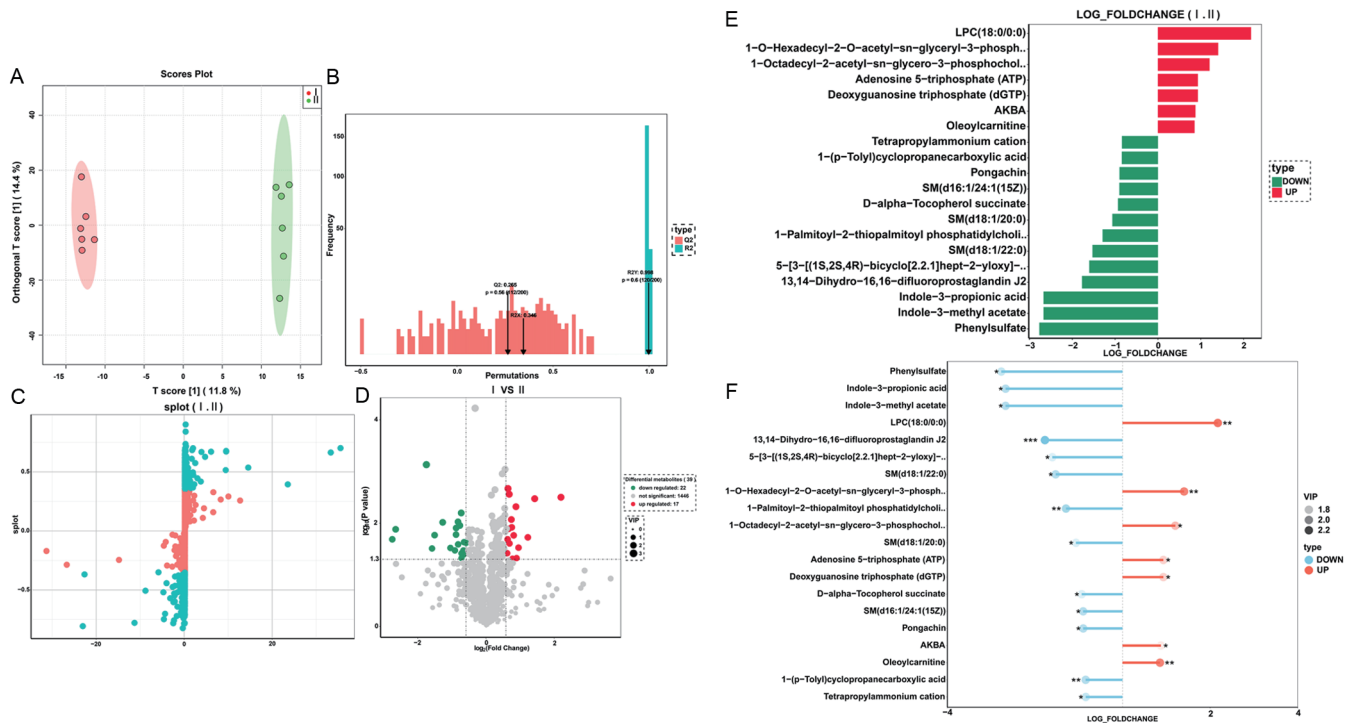


Fig. 7. Effect of TM6SF2 gut-specific knockout on serum metabolites in mice. (A) Orthogonal partial least-squares discriminant analysis plot for the serum metabolites comparison combination of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (B) Orthogonal partial least-squares discriminant analysis model validation plot for the comparison combination of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (C) Orthogonal partial least-squares discriminant analysis model Splot plot for each comparison combination of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (D) Identification of differentially metabolized compounds using a volcano plot; (E) Bar plot for the differential metabolites fold (presenting the top 20 differential metabolites with VIP value) between the serum metabolite combinations of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (F) Lollipop plot for the differential metabolites fold among the serum metabolite combinations of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice (the horizontal axis refers to the log2FC of the differential metabolites in the two comparison combinations, the color of the dots represents the VIP value, the top 20 differential metabolites with the largest absolute log2FC values are shown; I, TM6SF2 GKO-HFD mice, II, CON-HFD mice; and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

from previous studies. Notably, despite the lack of difference in serum TG levels, TM6SF2 GKO mice presented with significantly elevated hepatic TG levels under an HFD. This indicates that TM6SF2 may affect hepatic lipid retention. Previous investigations have demonstrated that TM6SF2 plays an important role in VLDL production in the liver.¹⁹ Thus, the increase in lipid accumulation in the liver of TM6SF2 GKO mice fed an HFD may be attributed to the impaired hepatic lipid export capacity. More importantly, the present findings revealed that the depletion of TM6SF2 in the intestines affected lipid metabolism in the liver, suggesting the role of TM6SF2 in the gut-liver axis.

Elevated serum ALT and AST levels have been widely accepted as indicators of liver damage, and these are generally used to assess liver function in MASLD.^{14,21} A recent study revealed that the TM6SF2 E167K variant is associated with elevated ALT levels and increased susceptibility to MASLD development.²² Consistently, it was observed in the present study that serum levels of ALT and AST significantly increased in TM6SF2 GKO mice compared to CON mice ($p < 0.05$), indicating increased liver lipid degeneration and susceptibility to MASLD. However, the molecular mechanism by which TM6SF2 affects lipid deposition in the liver requires further investigation.

Although the pathogenesis of MASLD remains not fully understood, dysbiosis of the gut microbiota and activation of the associated enterohepatic axis are currently recognized as key processes in the development of MASLD.²³ Using 16S rRNA gene sequencing, it was found that HFD significantly reduced

intestinal microbial diversity. More importantly, TM6SF2 GKO further decreased the richness of the intestinal flora under HFD. In alignment with these findings, clinical studies have reported that MASLD patients had significantly lower microbiota diversity compared to non-NAFLD subjects.²⁴ Interestingly, the present study found that HFD increased the Firmicutes/Bacteroidetes ratio in mice, while TM6SF2 GKO reduced this ratio compared to CON-HFD mice. This suggests that the cross-talk among diet, gut microbiota, and genetic variations plays critical roles in MASLD initiation and progression. The present findings are similar to prior studies from Asian populations.^{25–27} Tsai and colleagues reported that NAFLD patients had higher levels of Bacteroidetes and lower levels of Firmicutes in a cohort of 50 biopsy-proven NAFLD patients (25 with NAFL and 25 with NASH) and 25 biopsy-proven healthy subjects (non-NAFLD).²⁵ Similar results were reported by Wang *et al.*²⁶ and Shen *et al.*,²⁷ both of whom examined Asian populations. Interestingly, these results significantly differ from those of a previous study conducted in Western countries, which reported lower levels of Bacteroidetes in NASH patients.²⁸ The reason may be that Western diets are characterized by high levels of fat, protein, and sugar, while Asian diets are typically low in fat and protein but high in dietary fiber. Similarly, the Latino diet consists mostly of vegetables, nuts, grains, and low-fat red or processed meats.²⁹ It can be concluded that differences in dietary composition have a significant effect on the growth of Bacteroidetes. The present findings indicate that TM6SF2 GKO may aggravate MASLD development by affecting intestinal microbial diversity/com-

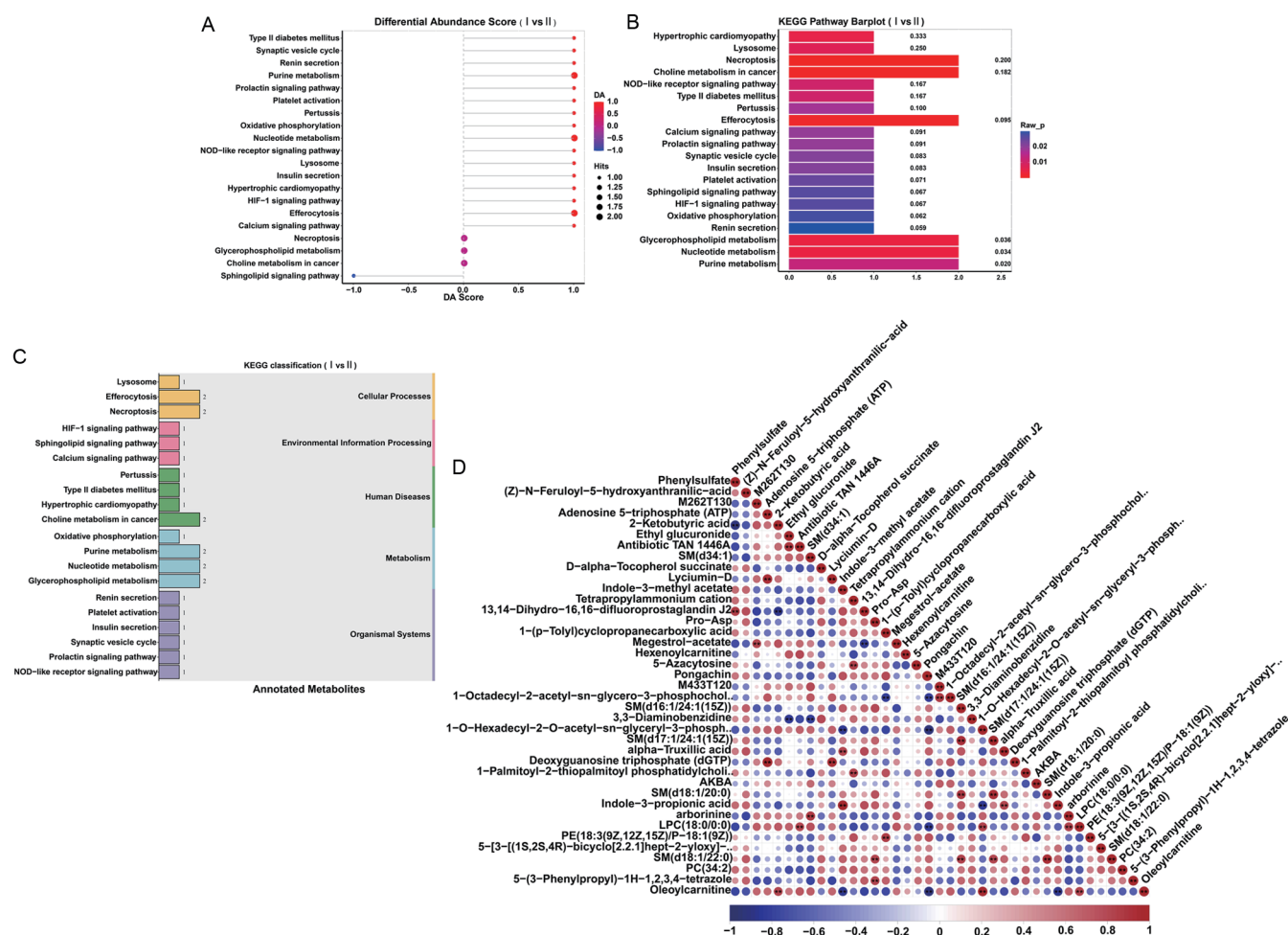


Fig. 8. Effect of TM6SF2 gut-specific knockout on the KEGG of blood metabolites. (A) Differential abundance score chart of serum metabolites in high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (B) KEGG enrichment bar chart for serum metabolites in high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (C) KEGG metabolic pathway classification bar chart for serum metabolites in high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; (D) Differences in metabolites correlation heat maps (red indicates a stronger positive correlation, and blue indicates a stronger negative correlation; I, TM6SF2 GKO-HFD mice, II, CON-HFD mice; * $p < 0.05$, ** $p < 0.01$).

position under identical dietary conditions. Furthermore, the present study highlights the role of genetic variations in the gut microbiome and NAFLD susceptibility.

Intestinal permeability plays an essential role in the gut-liver axis during MASLD pathogenesis. In the present study, Alcian blue staining results revealed an increase in intestinal permeability in HFD-fed mice compared to their CD-fed littermates. The increase in intestinal permeability can facilitate the translocation of gut bacteria and their metabolites to the liver via the portal vein system. This bacterial translocation to the liver may potentially promote hepatic steatosis, inflammation, and fibrosis.

To explore the alterations in metabolic-related TM6SF2 GKO, serum metabolites were analyzed by UHPLC-QE-MS. Under HFD conditions, 17 upregulated and 22 downregulated metabolites were detected in TM6SF2 GKO mice. Notably, the level of LPC (10:0/0:0), a bioactive lipid, was elevated in HFD-fed TM6SF2 GKO mice. Furthermore, KEGG pathway analysis revealed that choline metabolism in cancer and efferocytosis pathways were upregulated, while the sphingolipid signaling pathway was downregulated in TM6SF2 GKO mice under HFD conditions. Therefore, TM6SF2 GKO may alter the

diversity and abundance of the intestinal flora, thereby affecting the expression of metabolites in serum and aggravating MASLD through the "gut-liver axis".

The correlation analysis revealed significant associations among various metabolites. For instance, the level of LPC (18:0/0:0) was correlated with the level of Arborinine, 1-O-Hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine, and Antibiotic TAN, while Oleoylcarnitine content was correlated with 1446A and 2-Ketobutyric acid. Furthermore, a consistent correlation was observed between the differential gut microbiome genus and serum metabolites in HFD-fed mice. Receiver operating characteristic curve analysis revealed that LPC (18:0/0:0), Arborinine, Antibiotic TAN, 1446A, Oleoylcarnitine, and 2-Ketobutyric acid are potential serum biomarkers for MASLD exacerbation.

We acknowledge that our study has several limitations. First, we did not compare intestinal TM6SF2 expression between healthy individuals and MASLD patients. Future studies involving intestinal biopsies from diagnosed MASLD patients will be valuable to validate our results. Second, while our current data suggest the importance of intestinal TM6SF2 in MASLD pathogenesis, the underlying mechanism has not

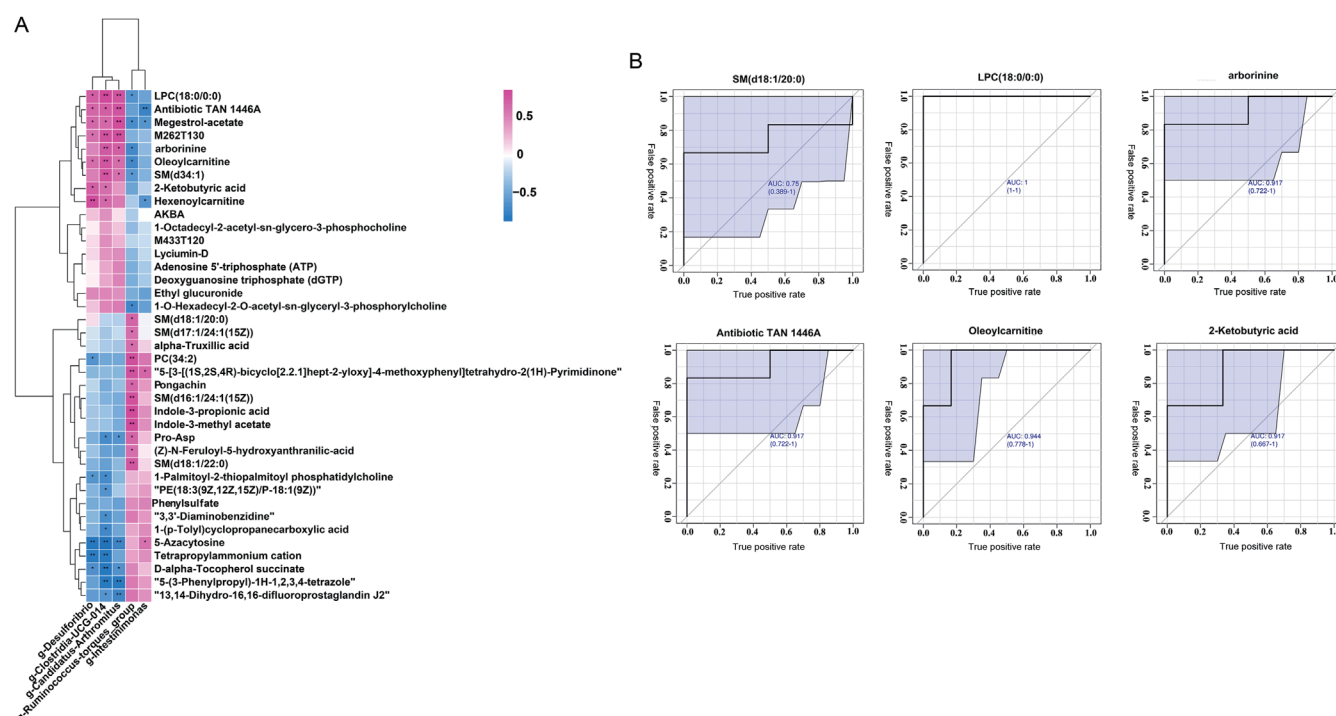


Fig. 9. Analysis of differential metabolites in serum. (A) High-fat diet-fed mice under the condition of the intestinal flora, differences, and the correlation heatmap analysis of differences among serum metabolites; (B) Effect of TM6SF2 gut-specific knockout on the ROC of differential metabolites (the ROC of differential metabolites in the serum metabolites of high-fat diet fed TM6SF2 gut-specific knockout and wild-type mice; the curves for SM[d18:1/20:0], LPC[18:0/0:0], Arborinine, 1-O-Hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine, Antibiotic TAN 1446A, Oleoylcarnitine, and 2-Ketobutyric acid; * $p < 0.05$, ** $p < 0.01$). ROC, receiver operating characteristic.

yet been fully dissected. However, our ongoing mechanistic investigation, including restorative experiments related to microbiota and changes in metabolites, will help strengthen these findings.

Conclusions

TM6SF2 GKO can aggravate liver lipid accumulation and liver injury in MASLD mice. Furthermore, TM6SF2 GKO regulates the gut microbiota and may aggravate MASLD through the "gut-liver axis".

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Conflict of interest

YNX has been an Editorial Board Member of the *Journal of Clinical and Translational Hepatology* since 2013. The other authors have no conflicts of interest related to this publication.

Author contributions

Study concept and design (LZC, YNX, CCM), acquisition of

data, experiments, analysis and interpretation of data (YRW, SLZ), drafting of the manuscript (LZC, YRW), and revision of the writing and project supervision (LZC, ZZZ, YNX, CCM). All authors made significant contributions to the study and approved the final manuscript.

Ethical statement

All animal experiments were performed in accordance with the guidelines approved by the Medical Laboratory Animal Ethics Committee of Qingdao Municipal Hospital (No. 2024-KY-005). All animals received human care.

Data sharing statement

The datasets used in the study are available from the corresponding author upon reasonable request.

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