Materials and Methods

Mice

All mice were housed in ambient room temperature (22 ± 1°C) with 12/12 h light-dark cycles and free access to water and food. Eight-weeks-old male C57BL/6J mice were fed either normal chow diet (ND; 12% energy from fat), HFD (60% energy from fat, Research Diets), MCDD (Dyets Inc.), or HFHCD (60% energy from fat containing 2.5% cholesterol, Dyets Inc.). In another group, mice were fed Western diet (TD.120330; 0.2% cholesterol + 22% hydrogenated vegetable oil, Envigo RMS, Inc.) supplemented with high fructose syrup in the drinking water.[1] After the indicated time of diet feeding, mice were fasted for 5 h in the morning before they were euthanized. Blood samples were collected for biochemical analysis, and stored at −80°C. To examine the role of cholesterol in the regulation of *Sms1* expression, we fed C57BL/6J mice cholesterol-enriched diet (CED, C18021901; 2% cholesterol and 0.5% sodium cholate, Research Diets) for two days.[2]

Caspase-1 K/O mice (#016621) and *Caspase-11* K/O mice (#024698) with a C57BL/6J genetic background were purchased from the Jackson Laboratory. *Nlrp3* K/O mice were generated using the transcription activator-like effector nucleases (TALEN) method.[3] *Nlrp3*-specific TALEN mRNAs were introduced into C57BL/6N mouse zygotes. To evaluate the non-specific effects of TALEN, potential off-target sites were predicted as previously described[4] and T7E1 assays were performed using the genomic DNA of F0 mice. The selected mutant allele was a 13-bp deletion in the exon 2 of *Nlrp3* that induces a frameshift in the reading frame followed by a premature stop codon. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Asan Institute for Life Sciences, Seoul, Korea.

Human liver tissues

Human liver samples were obtained from liver explants of donors and recipients diagnosed with NASH/cirrhosis undergoing liver transplantation at the Liver Transplantation Unit of the Hospital Clinic, Barcelona. During the donor sample collection, an assessment of the liver is performed to rule out steatosis, fibrosis or cirrhosis before transplantation. A biopsy of the resected liver from the recipient was performed right after the hepatectomy and samples were fixed in formalin for histological examination. The persons that entered the liver transplantation as donors but that the degree of fat infiltration precluded the use of the graft for ultimate transplantation were classified as having simple steatosis. Hepatic FC levels were determined by HPLC analyses as described previously.[5] Samples from subjects exhibiting steatosis were used to assess the expression of SMS1 and SMS2. The clinical data of the patients are presented in online supplementary Table S1. In addition, normal liver tissue was obtained from the surgical specimens of donor livers used for transplantation. The protocol was approved by the Hospital Clinic/UB Ethics Committee (HCB/2012/8011) of Hospital Clinic, Barcelona, Spain.

Isolation and culture of hepatocytes

After 4 weeks of control or HFHCD feeding, C57BL/6J mice were sacrificed to isolate the hepatocytes. Mice were anesthetized with pentobarbital sodium (Hanlim Pharm) by intraperitoneal injection and perfused with Hank's balanced salt solution (HBSS; Welgene) pre-warmed in a 42℃ water bath. Then, 0.36 mg/ml collagenase (Gibco) and trypsin inhibitor (Sigma-Aldrich) were immediately added before liver perfusion. After cleaning the abdomen under sterile conditions, the abdominal cavity was opened and the portal vein was cannulated with a 24 G catheter. Perfusion solution was infused using a pump at a flow rate of 5 ml/min. After starting the perfusion, the vena cava was incised to permit sufficient outflow. Correct placement of the portal vein catheter was confirmed during perfusion by the steady and even change in color from dark red-brown to light brown in all liver lobes. Following perfusion, the gallbladder was removed and the liver was

transferred to Dulbecco's modified Eagle's medium (DMEM; Welgene) medium containing 10% fetal bovine serum (FBS; Gibco). The subsequent steps were performed on a clean bench. The liver capsule was carefully removed using a scraper, and the suspension was placed onto a Falcon® 100-μm cell strainer (Corning Inc.) and was filtered by gravity flow. The suspension was transferred to a 50-ml conical tube and washed twice with DMEM. The cells were centrifuged at 50 × g for 3 min at room temperature. This pellet was resuspended in DMEM with 36% Percoll (Sigma-Aldrich) and then centrifuged at 50 × g for 10 min at room temperature. The pellet was then washed two times with PBS. Finally, the hepatocytes were plated on collagen-coated tissue culture dishes in DMEM cell culture medium supplemented with 10% FBS and 1% penicillin/streptomycin solution, and kept in a humidified cell culture incubator with 5% CO_2 at 37℃.[6 7]

Isolation and culture of Kupffer cells

Primary mouse Kupffer cells were isolated from 8-weeks-old mice by *in situ* collagenase perfusion and differential centrifugation on OptiPrep (Sigma-Aldrich) density gradients as described previously.[8] Mouse livers were perfused with HBSS solution with 0.36 mg/ml collagenase (Gibco). The homogenate was filtered through a 70-μm mesh and centrifuged at 50 × g for 5 min at room temperature to remove the hepatocytes. The supernatant was transferred to a new tube and centrifuged at 1600 rpm for 10 min at 4℃. The cell pellet was then resuspended in 4 ml of 20% OptiPrep, carefully loaded with 4 ml of 11.5% OptiPrep, and finally loaded with 5 ml of HBSS solution. The cell pellet was then centrifuged at 3000 rpm for 17 min at 4℃. The cell fraction between 20% and 11.5% OptiPrep gradient were gently collected in a new tube. After washing, the red blood cells (RBCs) were lysed with RBC lysis buffer (Sigma-Aldrich) and the final cell pellet was resuspended in RPMI 1640 medium (Welgene) containing 1% penicillin, streptomycin, and 10% FBS, and plated onto uncoated 24-well plates at a density of 1×10^4 cells per well. After 10 min, non-adherent cells and debris were removed by washing and replacing the medium.[9]

AAV-mediated gene transfer

Adeno-associated virus (AAV) vectors carrying *Sms1* (AAV8-GFP-U6-m-SGMS1- short hairpin RNA (shRNA), shAAV-271857), *Pkcδ* (AAV8-GFP-U6-m-PRKCD-shRNA, shAAV-269379), or *Nlrc4* (AAV8-GFP-U6-m-NLRC4-shRNA, shAAV-Cust: 5'-GCA CAG AAT CTT CAC AAT TTG TCA AGA GCA AAT TGT GAA GAT TCT GTG CTT TTT-3')-specific short hairpin RNA (shRNA) or control shRNA (AAV8-GFP-U6-scrmb shRNA, Control AAV: 5'-CAA CAA GAT GAA GAG CAC CAA CTC GAG TTG GTG CTC TTC ATC TTG TTG TTT TT-3') were obtained from Vector Biolabs. The vectors were intravenously injected into the tail vein of 8-weeks-old C57BL/6J mice at a dose of 4×10^{11} plaque-forming units per mouse. The mice were fed HFHCD after viral infection, and the same dose was injected again after 6 weeks.

Histological analysis

Liver tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. Hepatic fibrosis was analyzed by Masson's trichrome staining. To detect connective tissues, additional sections were stained with 0.1% Sirius Red in saturated picric acid. Images were captured using a microscope (Olympus).

Immunohistochemistry

For immunohistochemical analysis, paraffin-embedded liver sections were deparaffinized with xylene, rehydrated through a series of graded ethanol, and washed with PBS for 10 min. Slides were treated with 10 mM citrate buffer (pH 6.0, Sytek) for antigen retrieval and endogenous peroxidases were inactivated with peroxidase blocking solution (Dako) for 30 min. Samples were blocked with blocking buffer (Dako) for 30 min at room temperature and incubated with primary antibodies (F4/80 [Abcam, #ab6640, 1:50], α-SMA [Abcam, #ab7817, 1:100]) overnight at 4°C. Then, the slides were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rat IgG-HRP

[Biolegend, #405405, 1:200], anti-mouse IgG-HRP [Biolegend, #405306, 1:400]) for 30 min. All sections were counterstained with hematoxylin and the resulting images were captured using a microscope (Olympus). Images were quantified using the Image J Fiji software.

TUNEL staining

TUNEL staining was performed per the manufacturer's instructions (Abcam). Paraffin-embedded liver tissues were deparaffinized in xylene and then rehydrated in graded ethanol. Briefly, slides were perrmeabilized with proteinase K solution for 30 min, and endogenous peroxidases were inactivated by 3% H_2O_2 for 5 min. The slides were covered with TdT equilibration buffer for 30 min. Tissues were labeled with a TdT labeling reaction mixture for 1.5 h at 37°C, and the labeling reaction was terminated with stop buffer for 5 min. Then, the slides were blocked with blocking buffer for 30 min, and incubated in conjugate solution for 30 min for signal detection. DAB solution was added for 15 min, and the slides were washed with water. Slides were counterstained with hematoxylin (Sigma-Aldrich) for 1 min, dehydrated in graded ethanol, and mounted with mounting solution (Thermo Fisher Scientific).

Real-time PCR analysis

Total RNA (2 μg) was isolated using the Trizol reagent (Thermo Fisher Scientific Inc.) and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was performed using the SYBR green according to the manufacturer's instructions. Each reaction was run in triplicates to determine the threshold (Ct) of each mRNA, and the amount of each cDNA relative to the internal control Tbp or β-ACTIN was determined using the 2-ΔΔCt method. The primers were designed based

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509

on the nucleotide sequences in the GenBank database (online supplementary Table S2).

Western blot analysis

The liver and cells were lysed with protein lysis buffer (1% NP-40, 150 mM KCl, 50 mM Tris–HCl pH 7.4, 4 mM EDTA, 2 mM EGTA) supplemented with protease inhibitors (Roche Diagnostics) and phosphatase inhibitor (Roche Diagnostics). Proteins were subjected to immunoblotting with primary antibodies overnight at 4°C and horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Antibodies used in the present study are listed in online supplementary Table S3. The signal intensities of the protein bands were quantified with the Image J software and normalized using the intensity of the loading control.

Liver TG contents

TG content in the liver was determined in duplicate using the Sigma Triglyceride (MAK266) kit.

Plasma ALT measurement

Blood was collected from mice and centrifuged at 3000 rpm for 10 min at 4°C to obtain plasma. Plasma ALT levels were measured using the ALT Endpoint Assay kit (Abcam).

Measurement of lipid metabolites

We added 400 µl chloroform/methanol (2:1 ratio) and 100 µl internal standard solutions (500 nM of 17 ceramide, 1 µM of 19:0 DAG-d5, 200 nM of 18:0 D70 PC) to mouse liver (10–20 mg). The sample was homogenized using a TissueLyzer (Qiagen) and allowed to stand for 20–30 min at 4°C. The supernatant was collected after centrifugation for lipid extraction. We then added 400 µl H_2O to the supernatant, followed by vigorous vortexing. The bottom organic layer containing lipids was collected

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509

after centrifugation and transferred to a new tube. Liquid-liquid extraction was repeated if needed. The organic layer was dried under vacuum, and the dried matter was reconstituted with 20 µl methanol before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS equipped with 1290 HPLC (Agilent) and Qtrap 5500 (AB sciex) was used. MS was operated in the positive ion mode with a turbo ion-spray voltage of 5500 V using 20 psi curtain gas, 50 psi nebulizer gas, and 50 psi drying gas at 400°C. For the measurement of sphingolipid (ceramide, SM) and DAG, a reverse-phase column (Pursuit 5 C18 150 × 2.0 mm) was used. HPLC used the mobile phase A (5 mM ammonium formate/MeOH/tetrahydrofuran; 500:200:300) and mobile phase B (5 mM ammonium formate/MeOH/tetrahydrofuran; 100:200:700) and proceeded at 200 µl/min in 35°C. The separation gradient for sphingolipid was as follows: 50% of A at 0 min, 50% of A for 5 min, 50–30% of A for 3 min, 30% of A for 7 min, 30–10% of A for 7 min, 10% of A for 3 min, 10–50% of A for 0.1 min, then 50% of A for 4.9 min. The separation gradient for DAG was as follows: 10% of A at 0 min, 10% of A for 6.5 min, 10-5% of A for 0.1 min, 5% of A for 3.4 min, 5–10% of A for 0.1 min, and then 10% of A for 1.9 min.

For the measurement of PC, a reverse-phase column (Zorbax Eclipse Plus C18 50 × 2.1 mm, 1.8 µm) was used. The separation gradient used mobile phase A (10 mM ammonium acetate in MeOH/isopropanol/H2O; 900:50:50) and mobile phase B (10 mM ammonium acetate in MeOH/isopropanol/H2O; 940:50:10) and proceeded at 400 µl/min and 25°C. The separation gradient was as follows: 50% of A at 0 min, 50% of A for 5 min, 50–20% of A for 5 min, 20% of A for 17 min, 20–50% of A for 0.1 min, and then 50% of A for 2.9 min.

Multiple reaction monitoring mode was used in the positive ion mode, and the extracted ion chromatogram corresponding to the specific transition for each lipid was used for quantification. The calibration range for sphingolipids was 0.1–1000 nM ($r^2 \geq 0.99$). For PCs, the peak area ratio of each lipid/internal standard was used for relative comparison.

Luciferase assay of *Sms1* promoter activity

Mouse *Sms1* promotor clone (MPRM 48214-PG02; Genecopoeia Inc.) was subcloned into the pEZX-LvPG04 vector (Genecopoeia Inc.). AML12 (ATCC) cells were seeded in 24-well plates and transduced with *Sms1* promoter in the presence of Lipofectamine 3000 (Invitrogen) for 6 h. After 48 h of transfection, the cells were treated with DMSO or water-soluble cholesterol (Sigma-Aldrich) with avasimibe (Sigma-Aldrich) at different concentrations. After 3 h, culture media were harvested and analyzed with Secrete-Pair™ Gaussia Luciferase Dual Luminescence Assay kits (Genecopoeia Inc.) using the VICTOR X2 luminescence luminometer (Perkin-Elmer Wallance), according to the manufacturer's instructions.

Measurement of mitochondrial DNA

DNA was isolated from collected media with a total DNA extraction kit (iNtRON Biotechnology). The levels of mtDNA encoding Nd1, Cox2, and Atp6 were measured by qPCR with the same volume of the DNA solution. The following primers were used: mouse Nd1 forward, 5’-TAT CCA CGC TTC CGT TAC GA-3’, and reverse, 5’- GGT GGT ACT CCC GCT GTA AA-3’; mouse Cox2 forward, 5’-ACC GAG TCG TTC TGC CAA TA-3’, and reverse, 5’- GCT TGA TTT AGT CGG CCT GG-3’; and mouse Atp6 forward, 5’- GCC GTA ATT ACA GGC TTC CG-3’, and reverse, 5’- GTA AGC CGG ACT GCT AAT GC-3’.

Hepatic FC measurement

Hepatic FC level (Applygen Technologies Inc.) was measured by enzymatic methods according to the manufacturer’s instructions.

AML12 Cell culture

A mouse hepatocyte cell line, AML12, was cultured in DMEM (nutrient mixture F-12 1:1; Gibco) supplemented with 10% FBS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone in 37℃ in a humidified atmosphere of 5% CO_2 and 95% air.

Cell culture supernatant analysis

Primary hepatocytes were cultured for 48 h at 37°C. The supernatant was transferred and centrifuged at 1200 rpm for 10 min in 4°C to eliminate the debris. The level of lactate dehydrogenase (LDH) released in the supernatant was measured with the Cytotoxicity Detection Kit Plus (Roche Diagnostics). ATP levels were determined using a bioluminescent ATP assay kit (Biovision Inc.) according to the manufacturer's instructions, and the concentration of HMGB1 was measured using an ELISA kit (LSBio).

SMS1 overexpression

To generate a vector for overexpressing *Sms1*, mouse liver cDNA fragments of *Sms1* were amplified by PCR (SMS1 ORF-F: CCG GAA TTC ACC ATG TTG TCT GCC AGG AC, SMS1 ORF-R: CGC GGA TCC TTA TGT GTC GTT TAC CAG CC). The PCR products were subcloned into the pCDH-MCS lentiviral vector (CD513B-1, System Biosciences). Each plasmid was transfected in Lenti-X 293T cells (Clontech) along with packaging plasmids (pCMV-VSV-G, pMDLg/PRRE, and pRSV-Rev; 8454, 12251, 12253, Addgene) using Lipofectamine 3000. Lentiviruses were infected with 8 μg/ml of polybrene for 48 h. 5×10^5 AML12 cells were seeded into wells of a 6-well plate containing antibiotic-free medium the day before infection. SMS1 overexpression or negative control lentivirus infected for 36h, and then cells were harvested.

Isolation of extracellular vesicles (EVs) from hepatocyte-conditioned media

For isolation of EVs, primary hepatocytes were seeded onto 6-well plates and cultured for 3 h in DMEM containing 3% EV-free FBS (System biosciences). EVs were isolated from the primary hepatocyte-conditioned media by using the ExoQuick-TC kit (System biosciences). First, cells and debris were removed by centrifugation at 3000 × g for 15 min. The resulting supernatant was

transferred to a new tube; then, an appropriate volume of ExoQuick-TC was added and mixed by inverting for overnight at 4°C. The mixture was centrifuged at 1500 × g for 30 min at 4°C; in order to collect the exosomes in the bottom of the vessels, the supernatant was removed and the tubes were centrifuged again at 1500 × g for 30 min to remove any remaining supernatant. Finally, the pellet containing the exosomes was resuspended using DPBS without calcium and magnesium (Gibco) or specific buffer according to the downstream application.

Measurement of exosomes by flow cytometry

The isolated exosomes were detected by flow cytometry using BD FACS Canto II (BD Bioscience). The exosome gate was established based on light scattering and forward scatter using flow cytometry sub-micron particle size reference kit (Invitrogen), then defining the exosomes as events at 30 nm–100 nm. The isolated exosomes were then subjected to fluorescent labeling using PKH67 dye (Sigma-Aldrich), which is a green fluorescent dye that labels lipid membranes. Exosomes in DPBS were resuspended in 1 ml of PKH67 and diluent C mixture, incubated for 5 min, and stopped by adding an equal volume of 1% EV-free FBS including DPBS for 1 min. Data were analyzed with the FlowJo software.

Activation of NLRP3 inflammasome in Kupffer cells by DAMPs released by pyroptotic hepatocytes

Four weeks after HFHCD feeding, primary hepatocytes were isolated and incubated for 48 h in 6-well plates and the media were collected. Cell debris was removed by centrifugation at 300 × g for 10 min. Primary Kupffer cells from 8-weeks-old C57BL6/J mice were isolated and cultured in 24-well plates. Primary Kupffer cells were primed with 10 ng/ml LPS for 4 h, then washed with PBS and treated with

0.2 ml of conditioned medium in primary hepatocyte. After 30 min, the medium was collected and IL-1β in the medium was quantified using an ELISA kit (R&D Systems).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Unpaired two-tailed Student's *t*-tests were used to compare the two groups. One-way ANOVA was used to compare multiple groups. Bonferroni correction was applied for post hoc analysis of the multiple comparisons. All statistical tests were conducted according to two-sided sample sizes and were determined based on previous experiments using similar methodologies. In all experiments, all replicates were biological replicates. Statistical analysis and graphing were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp) or GraphPad Prism 7 (GraphPad Software).

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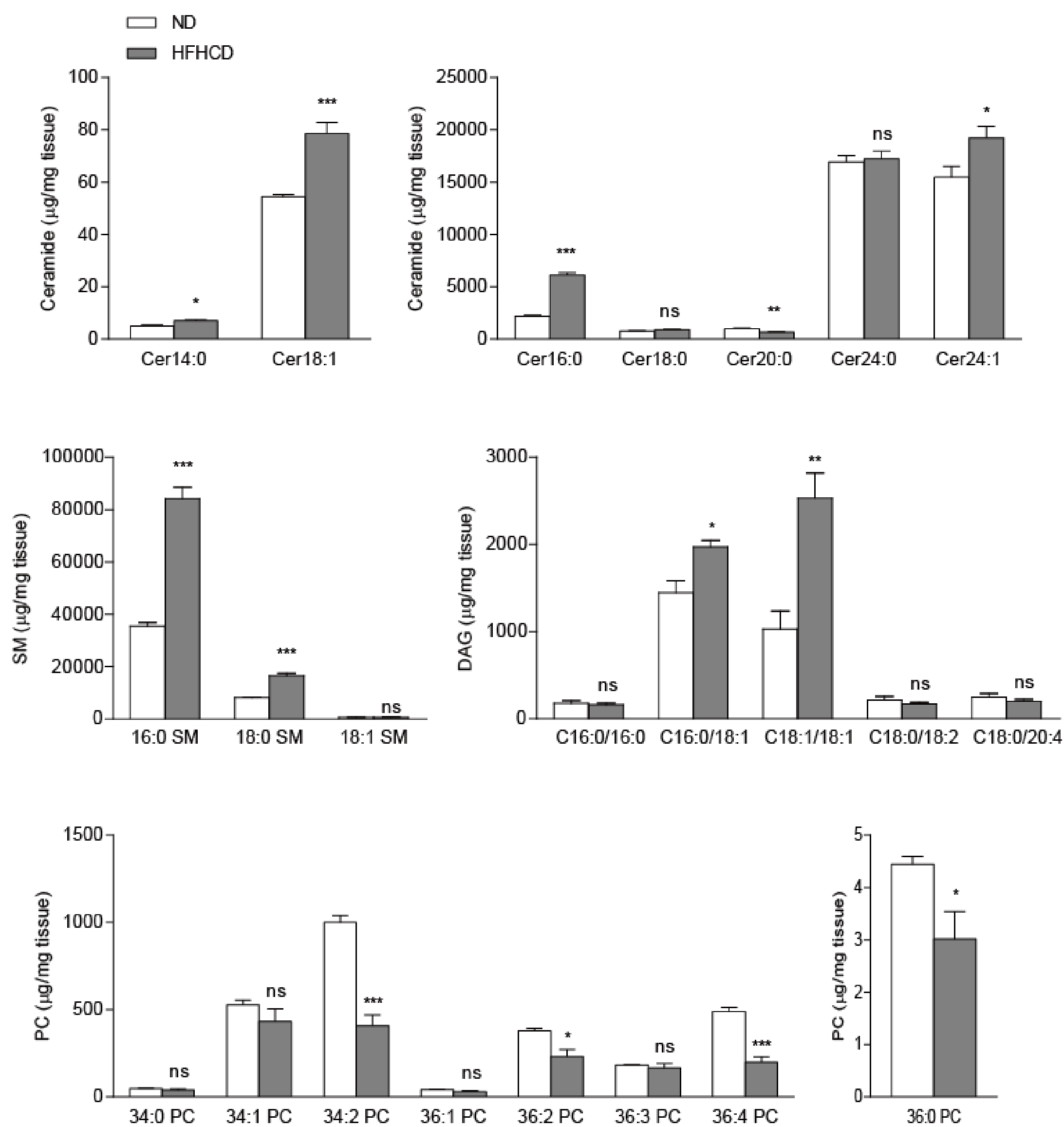
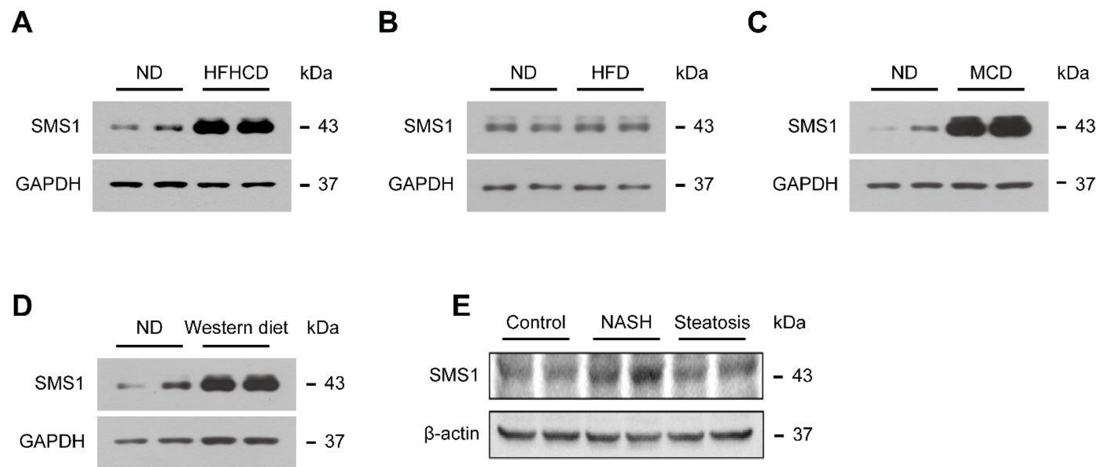
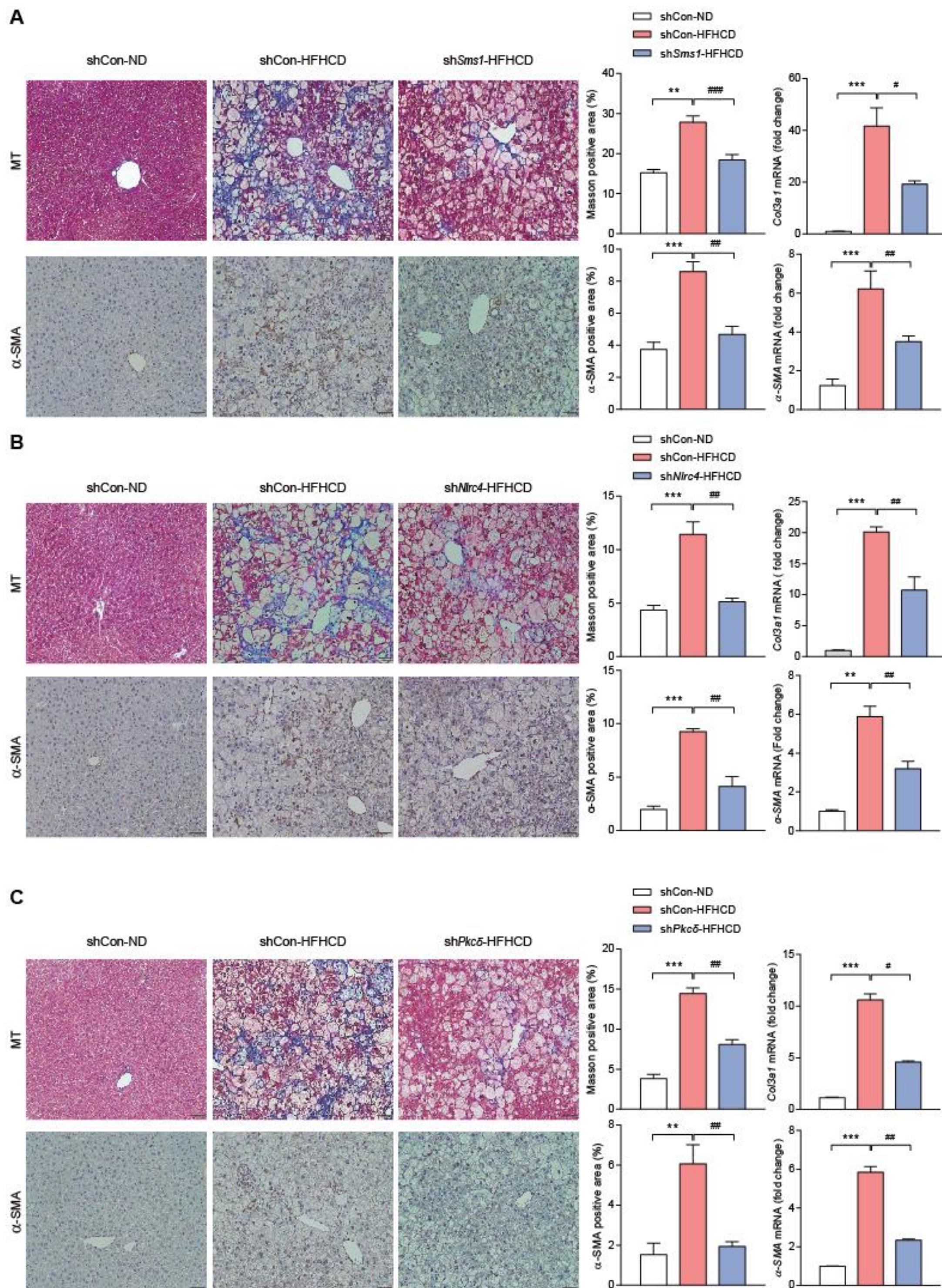


Supplementary Figure S1 Changes in the subset of lipid metabolites in the livers of HFHCD-fed mice. Levels of ceramide, SM, DAG, and PC subset in the liver of HFHCD-fed mice were determined by LC/MS/MS. Data are presented as mean ± SEM (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control diet (ND) mice; ns, not significant.

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509

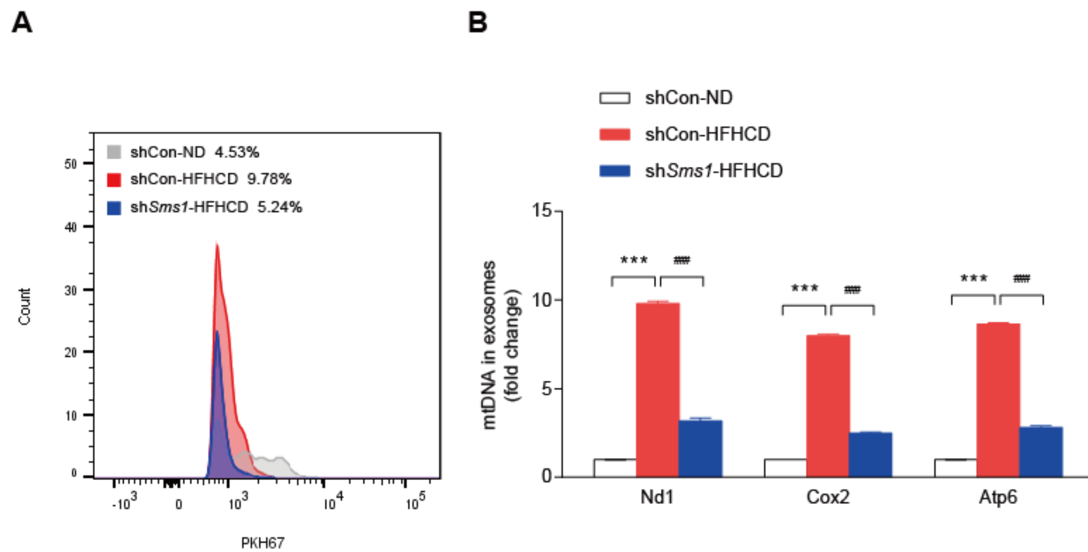


Supplementary Figure S2 SMS1 protein expression in the liver. Liver tissues were obtained from mice fed control diet (ND), HFHCD (A), or HFD (B) for 12 weeks. (**C**) SMS1 expression was observed in the livers of mice fed (C) control diet (ND) or MCDD for 8 weeks and (D) ND or Western diet for 16 weeks (n = 7). (E) SMS1 protein expression from the group of patients with NASH/cirrhosis or steatosis (n = 11-12).

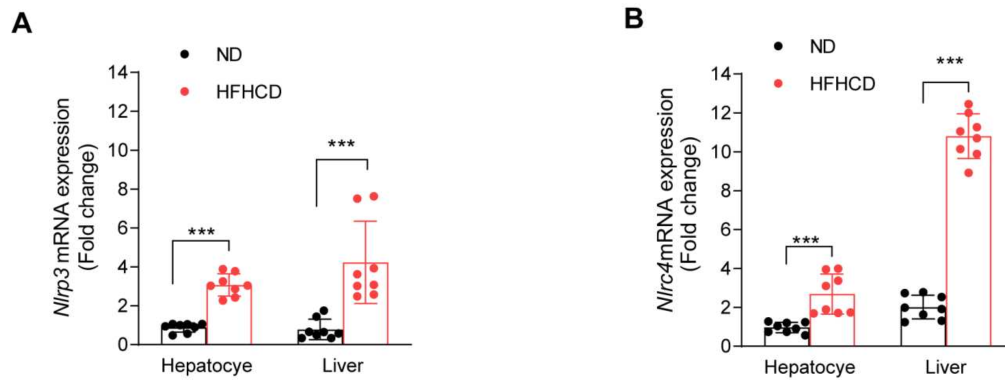

A
shCon-ND
shCon-HFHCD
shSms1-HFHCD
MT
α-SMA
Masson positive area (%)
Col3a1 mRNA (fold change)
α-SMA positive area (%)
α-SMA mRNA (fold change)
B
shNlrc4-HFHCD
α-SMA mRNA (Fold change)
C
shPkcδ-HFHCD

Supplementary Figure S3 Knockdown of *Sms1*, *Nlrc4*, or *Pkcδ* prevents hepatic fibrosis. Mice were injected with AAV carrying control shRNA (shCon), *Sms1*-specific shRNA (sh*Sms1*), *Nlrc4*-specific shRNA (sh*Nlrc4*), or *Pkcδ*-specific shRNA (sh*Pkcδ*) and then fed ND or HFHCD for 12 weeks. Effects of shRNA-mediated knockdown of (A) *Sms1*, (B) *Nlrc4*, and (C) *Pkcδ* on hepatic fibrosis are shown. Panels on the left show the representative MT staining and IHC of α-SMA in the liver, and bar graphs on the right show the quantification of MT and α-SMA staining, and the mRNA expression levels of *Col3al* and *α-Sma*. MT staining images in (A) are from Fig. 3A and those in (B) are from Fig. 5B. Data are presented as mean ± SEM (n = 5). $^{**}p < 0.01$ versus control mice, $^{***}p < 0.001$ versus control mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus shCon-HFHCD mice.

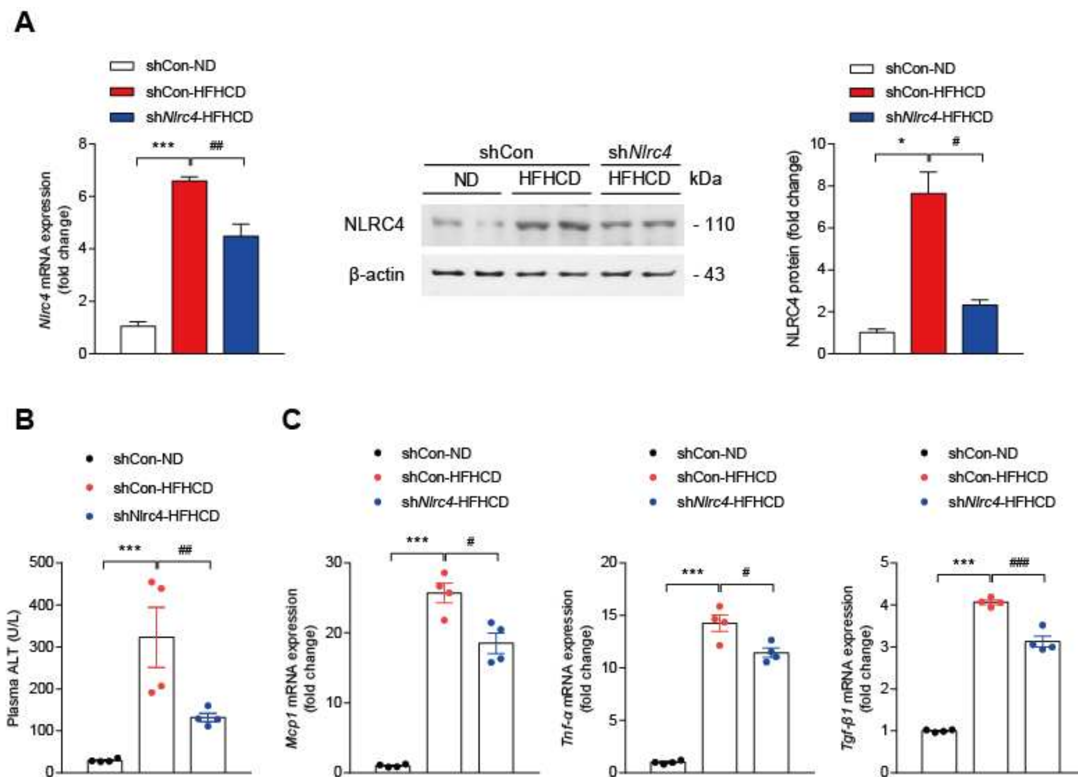
Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509



Supplementary Figure S4 SMS1 knockdown reduces mtDNA in the EVs from hepatocytes. Mice were infected with AAV carrying control shRNA (shCon) or *Sms1*-specific shRNA (sh*Sms1*) and then fed with ND or HFHCD for 4 weeks. EVs were isolated from primary hepatocyte-conditioned media. (A) Confirmation of EVs in the hepatocytes. Flow cytometry analysis of PKH67-positive particles in exosomes isolated from primary hepatocytes. (B) mtDNA contents in the EVs. Data are presented as mean ± SEM (n = 5). $^{***}p < 0.001$ versus control mice. $^{###}p < 0.001$ versus shCon-HFHCD mice.

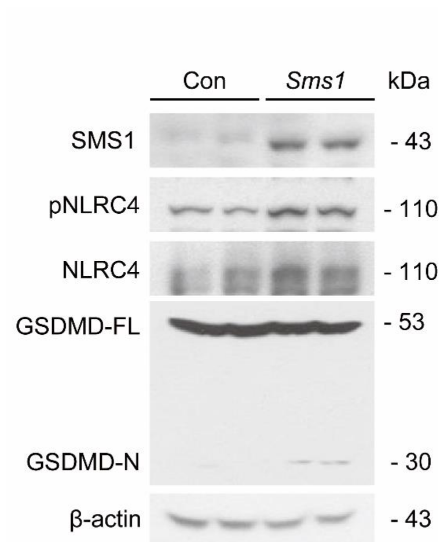


Supplementary Figure S5 HFHCD increases *Nlrp3* and *Nlrc4* mRNA expression in hepatocytes and the liver. Primary hepatocytes and liver were obtained from mice fed ND or HFHCD for 4 weeks and 12 weeks, respectively. *Nlrp3* (A) and *Nlrc4* (B) mRNA expression. Data are presented as mean ± SEM (n = 8). $^{***}p < 0.001$ versus control mice.



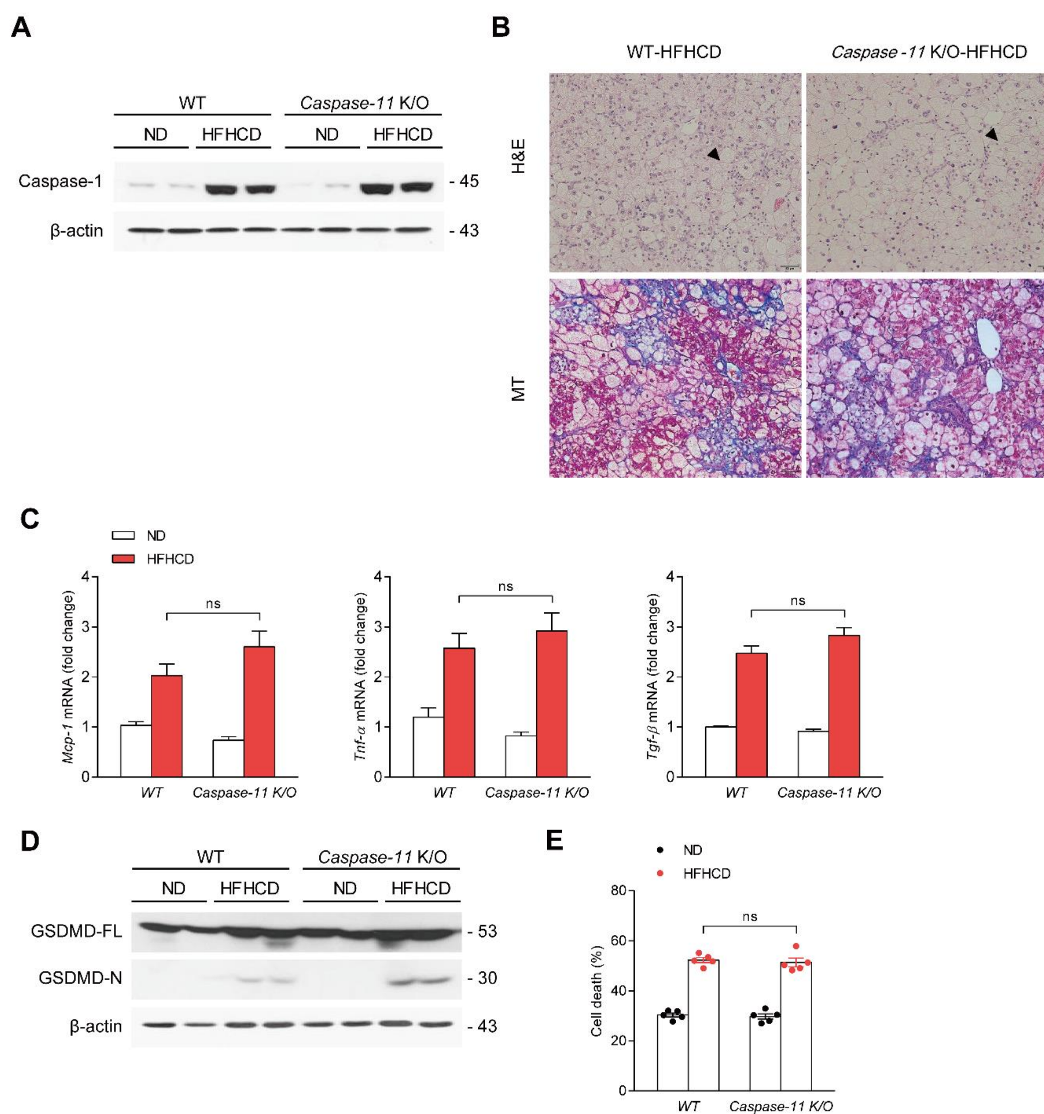
Supplementary Figure S6 *Nlrc4* knockdown prevents HFHCD-induced NASH development. Mice were infected with AAV carrying control shRNA (sh*Con*) or *Nlrc4*-specific shRNA (sh*Nlrc4*) fed ND or HFHCD for 12 weeks. (A) mRNA expression of *Nlrc4* in the liver and the representative Western blots of NLRC4 and the corresponding quantification. (B) Plasma ALT levels. (C) mRNA expression levels of *Mcp1*, *Tnf-α*, and *Tgf-β1*. Data are presented as mean ± SEM ($n = 4$). $^{*}p < 0.05$, $^{***}p < 0.001$ versus control mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus shCon-HFHCD mice.

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509



Supplementary Figure S7 SMS1 overexpression induces hepatocyte pyroptosis. Representative Western blots of SMS1, NLRC4, GSDMD-FL and GSDMD-N in AML12 cells (n=4). After *Sms1* overexpression, the cells were harvested 36 h later.

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509



Supplementary Figure S8 Caspase-11 does not play a major role in hepatocyte pyroptosis or NASH development. *Caspase-11* K/O mice were fed with ND or HFHCD for 12 weeks (A-C) or 4 weeks (D, E). (A) Representative Western blots of caspase-1 in liver tissues. (B) Representative H&E and MT staining in liver tissues. Scale bar, 50 μm. Arrowhead indicates inflammatory foci. (C) Relative mRNA expression levels of *Mcp1*, *Tnf-α*, and *Tgf-β1* in the liver. (D) Representative Western blots of GSDMD-FL and GSDMD-N in hepatocytes of wild type (WT) and *Caspase-11* K/O mice.

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509

(E) Cell death as measured by LDH levels. Data are presented as mean ± SEM (n = 5). ns, not significant.

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509

Supplementary Table 1. Clinical & analytical characteristics of patients with NASH/cirrhosis undergoing liver transplantation controls (donors, D), and steatosis (steatosis> 50%, SS).

Sample Patients	**Age (years)**	**Gender (M/F)**	**Weight (Kg)**	**BMI (Kg/m²)**	**AST (IU/L)**	**ALT (IU/L)**	**GGT (IU/L)**	**ALP (IU/L)**	**Bilirubin (mg/dl)**	**INR**	**Total Chole-sterol (mg/dl)**	**TG (mg/dl)**
D_5	42	M	90	27.8	30	30	30	NA	NA	1.0	NA	NA
D_22	67	M	85	29.4	NA	NA	NA	NA	NA	NA	NA	NA
D_20	70	F	NA	NA	NA	NA	18	NA	NA	1.1	NA	NA
D_39	74	M	70	25.7	NA	NA	NA	NA	NA	NA	NA	NA
D_40	50	M	62	22.8	117	62	NA	NA	NA	NA	NA	NA
D_80	76	M	75	27.5	20	35	21	NA	NA	1.0	NA	NA
D_8	34	M	75	23.1	30	23	28	NA	NA	1.1	NA	NA
D_263	75	F	78	28.7	19	16	11	NA	NA	NA	NA	NA
D_288	28	M	58	17.9	50	30	23	NA	NA	1.0	NA	NA
D_315	58	M	90	31.1	5	23	36	NA	NA	NA	NA	NA
D_355	74	M	82	26.8	9	12	40	NA	NA	1.0	NA	NA
NASH_8	46	M	78	26.1	18	76	735	298	1.2	4.68	192	212
NASH_38	59	M	68	26.6	44	33	19	109	9.9	1.79	66	41
NASH_140	57	M	93	28.7	20	13	23	203	1.3	2	58	45
NASH_175	56	F	69	27.3	18	6	42	116	0.6	1.36	183	172
NASH_237	65	M	63	23.7	41	35	215	193	2.1	1.36	123	87
NASH_239	49	M	91	34	58	10	257	141	4.8	1.72	144	84
NASH_258	60	F	100	35.9	45	25	183	152	1.4	1.4	79	70
NASH_269	67	M	95	33.7	37	20	76	199	3.2	1.4	59	47

Koh EH, *et al. Gut* 2020;0:1–11. doi: 10.1136/gutjnl-2020-322509

NASH_284	56	M	86	28.1	47	17	251	164	1.5	1.6	54	57
NASH_292	58	F	95	33.3	27	11	27	92	3.7	1.9	39	29
NASH_330	51	F	82	31.6	51	22	98	125	2.7	1.3	148	91
NASH_344	64	F	79	32.9	105	68	20	270	5.3	2.1	140	53
SS_7	78	F	60	21.3	20	22	NA	NA	NA	NA	NA	NA
SS_21	55	M	87	27	30	17	43	NA	NA	NA	NA	NA
SS_27	54	F	80	25	144	50	27	NA	NA	NA	NA	NA
SS_47	46	F	105	31	55	30	30	NA	NA	NA	NA	NA
SS_68	62	F	75	29	25	36	40	NA	NA	NA	NA	NA
SS_77	24	M	70	20	16	18	40	NA	NA	NA	NA	NA
SS_137	66	M	75	26	89	140	54	NA	NA	NA	NA	NA
SS_148	48	F	76	27	40	73	100	NA	NA	NA	NA	NA
SS_176	60	M	80	26	14	30	NA	NA	NA	NA	NA	NA
SS_178	46	M	75	25	23	35	39	NA	NA	NA	NA	NA
SS_183	66	M	65	27	183	135	48	NA	NA	NA	NA	NA
SS_189	47	F	100	31	16	12	11	NA	NA	NA	NA	NA

Supplementary Table 2. The Real-time PCR primer sequences and the DNA target sequences of shRNAs**.**

Gene	**Species**	**Forward (5'-3')**	**Reverse (5'-3')**
Real-time PCR primer sequences			
Tbp	Mouse	CCTTCACCAATGACTCCTATGAC	CAAGTTTACAGCCAAGATTCAC
Nlrp3	Mouse	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
Spt2	Mouse	AGCTTCGGTGCTTCAGGAGG	TCCATCACAGGCGGTGACAT
Sms1	Mouse	TCCATCACAGGCTCGCACA	ACGCTGAGGAGCCAGCAAA
Sms2	Mouse	TCCTCTTCAGCGGCCACAC	ATGCAGATGATCCCAGCCG
Mcp1	Mouse	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Tnf-α	Mouse	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
Tgf-β1	Mouse	TATAGCAACAATTCCTGGCG	CCTGTATTCCGTCTCCTTG
Nlrc4	Mouse	GAAACACTGTACGATCAGCTCC	CATGTTCTTGAAGCGATGGTTTT
Pkcδ	Mouse	CCTCCTGTACGAAATGCTCATC	GTTTCCTGTTACTCCCAGCCT
α-Sma	Mouse	ACTGGGACGACATGGAAAAG	GTTCAGTGGTGCCTCTGTCA
Col3a1	Mouse	GGGTTTCCCTGGTCCTAAAG	CCTGGTTTCCCATTTTCTCC
SMS1	Human	CATTTCAACTGTTCTCCGAAGC	CCATAGTGTGATACCACCAG
SMS2	Human	TTAATCTGCTGGCTGCTGAG	ACCAATCTTCTGAACCCGTG
β-ACTIN	Human	TTGCCGACAGGATGCAGAA	GCCGATCCACACGGAGTACT
cDNA target sequences of shRNAs			
Control shRNA	Mouse	CAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT	

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Sms1 shRNA	Mouse	CGGAGAATAATGAAGCTCATTCTCGAGAATGAGCTTCATTATTCTCCG	
Pkcδ shRNA	Mouse	CCTCACCGATTCAAGGTTTATCTCGAGATAAACCTTGAATCGGTGAGG	
Nlrc4 shRNA	Mouse	GCACAGAATCTTCACAATTTGTCAAGAGCAAATTGTGAAGATTCTGTGCTTTTT	
Gene/strand-specific PCR primer sequences			
Sms1 promoter	Mouse	ACGGTATCGATTCCTCCGTCGCCGGGAATG	CCGCTCGAGCGGCCGCTTAGTCACCACCG
Sms1 ORF	Mouse	CCGGAATTCACCATGTTGTCTGCCAGGAC	CGCGGATCCTTATGTGTCGTTTACCAGCC

Supplementary Table S3. Antibodies used in this study.

Antibody	Vender	Catalog number	Dilution factor
Caspase-1	AdipoGen	AG-20B-0042-C100	1:1000
NLRP3	AdipoGen	AG-20B-0014-C100	1:1000
P-NLRC4	ECM Biosciences	NP5411	1:1000
NLRC4	Invitrogen	PA5-72908	1:1000
P-PKCδ	Cell Signaling Technology	2055	1:1000
PKCδ	Cell Signaling Technology	2058	1:1000
GSDMD	Santa Cruz Biotechnology	sc-393656	1:1000
P-MLKL	Cell Signaling Technology	62233	1:1000
MLKL	Cell Signaling Technology	28640	1:1000
SMS1	Santa Cruz Biotechnology	sc-67097	1:1000
F4/80	Abcam	ab6640	1:50
α-SMA	Abcam	ab7817	1:100
β-actin	Sigma-Aldrich	A5441	1:20000
anti-rabbit IgG-HRP	BioLegend	406401	1:3000 - 1:5000
anti-mouse IgG-HRP	BioLegend	405306	1:3000 - 1:15000
anti-rat IgG-HRP	BioLegend	405405	1:200