

Supplementary Information

Targeting lipidated amino acid deficiency mitigates fibrosis via a PPAR α /CCL2 axis in metabolic dysfunction-associated steatohepatitis models

Sumit Kumar Anand^{1,19}, Sandeep Das^{1,19}, Fabio Arias^{1,19}, Koral S. E. Richard¹, Sumati Rohilla¹, Alia Ghrayeb^{1,2}, M. Peyton McKinney¹, Lu Wang³, Lin Tan⁴, Jibin Ding⁴, Dhananjay Kumar⁵, Nilesh Pandey¹, Jennifer Lee¹, Ying Zhao⁶, Suman Mohajan⁷, Gurranna Male⁷, Kelley Nunez⁸, Alexandra C. Finney¹, Brenna H. Pearson-Gallion^{4,9}, Reethika Gade¹, Jemiah Maxie⁴, Yu Liu^{10,11}, Harpreet Kaur¹, Rajan Pandit⁵, Cyrine Ben Dhaou¹, Joseph Eniafe¹², Xiaolu Zhang¹³, Zhipeng Liu¹⁴, Andrew D. Yurochko¹², Minerva T. Garcia-Barrio⁶, Babak Razani¹⁵, Fei Chang¹⁶, Francisco J. Schopfer¹⁶, Xin Huang¹⁷, Bishuang Cai^{10,11}, Eyal Gottlieb⁵, Bo Wen³, Duxin Sun³, Wanqing Liu¹⁸, Paul T. Thevenot⁸, Ari J. Cohen⁸, Jifeng Zhang⁶, Y. Eugene Chen⁶, A. Wayne Orr^{1,5}, Nirav Dhanesha¹, Arif Yurdagul Jr.^{1,5}, Oren Rom^{1,5,20}

¹ Department of Pathology and Translational Pathobiology, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA, USA

² Department of Cancer Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

³ Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, Ann Arbor, MI, USA

⁴ Metabolomics Core Facility, Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

⁵ Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA, USA

⁶ Department of Internal Medicine, Frankel Cardiovascular Center, University of Michigan Medical School, Ann Arbor, MI, USA

⁷ Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA, USA

⁸ Institute of Translational Research, Ochsner Clinic Foundation, New Orleans, LA, USA

⁹ Department of Biology, Centenary College of Louisiana, Shreveport, LA 71103, USA

¹⁰ Vatche and Tamar Manoukian Division of Digestive Diseases, Department of Medicine, University of California Los Angeles, Los Angeles, CA, USA.

¹¹ Comprehensive Liver Research Center at UCLA, University of California, Los Angeles, CA, USA

¹² Department of Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA, USA

¹³ Bioinformatics and Modeling Core, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA, USA

¹⁴ Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue University, West Lafayette, IN, USA

¹⁵ Division of Cardiology and Vascular Medicine Institute, Department of Medicine, University of Pittsburgh School of Medicine and University of Pittsburgh Medical Center, Pittsburgh, PA, USA

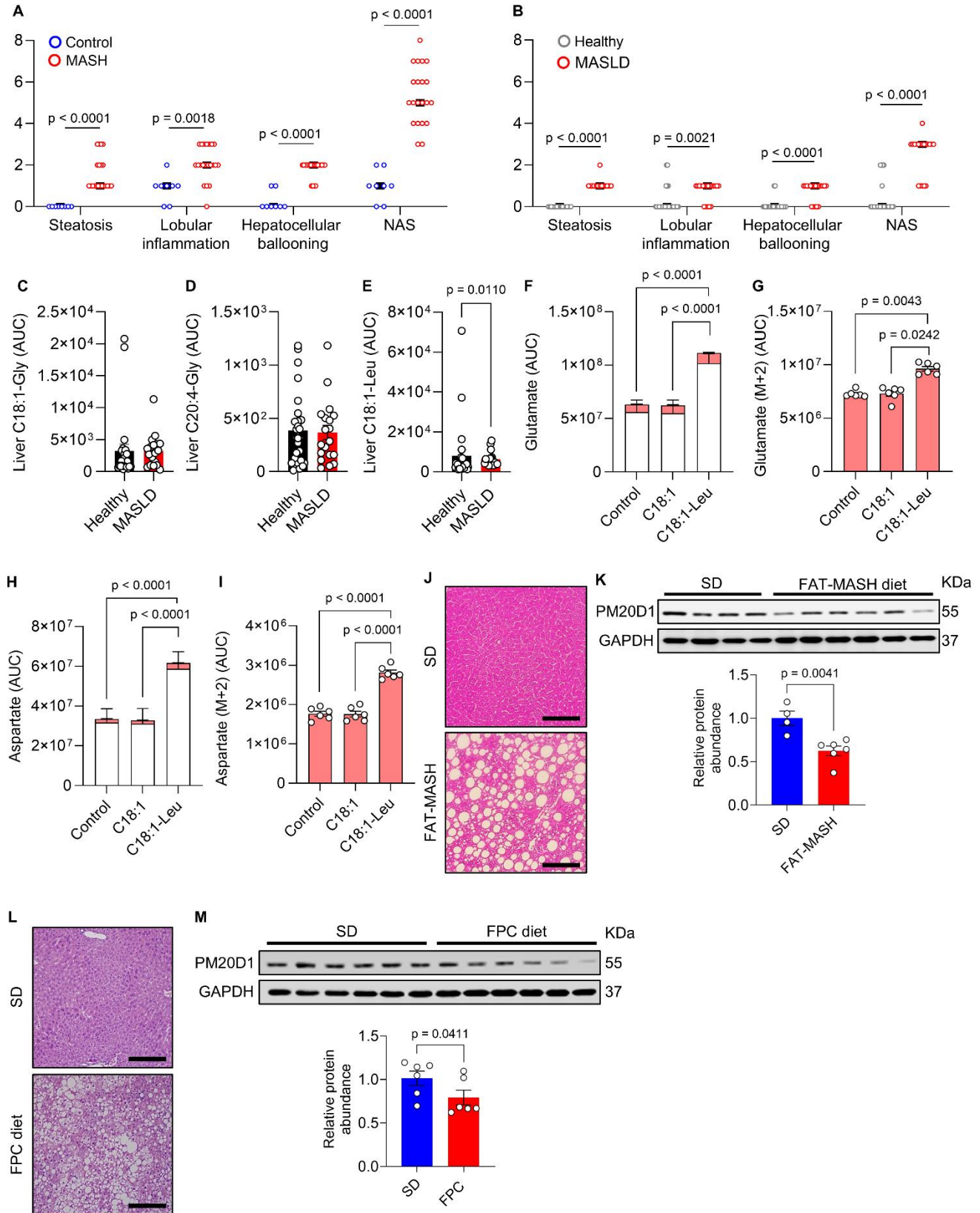
¹⁶ Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

¹⁷ Division of Hematology-Oncology, Department of Medicine and Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

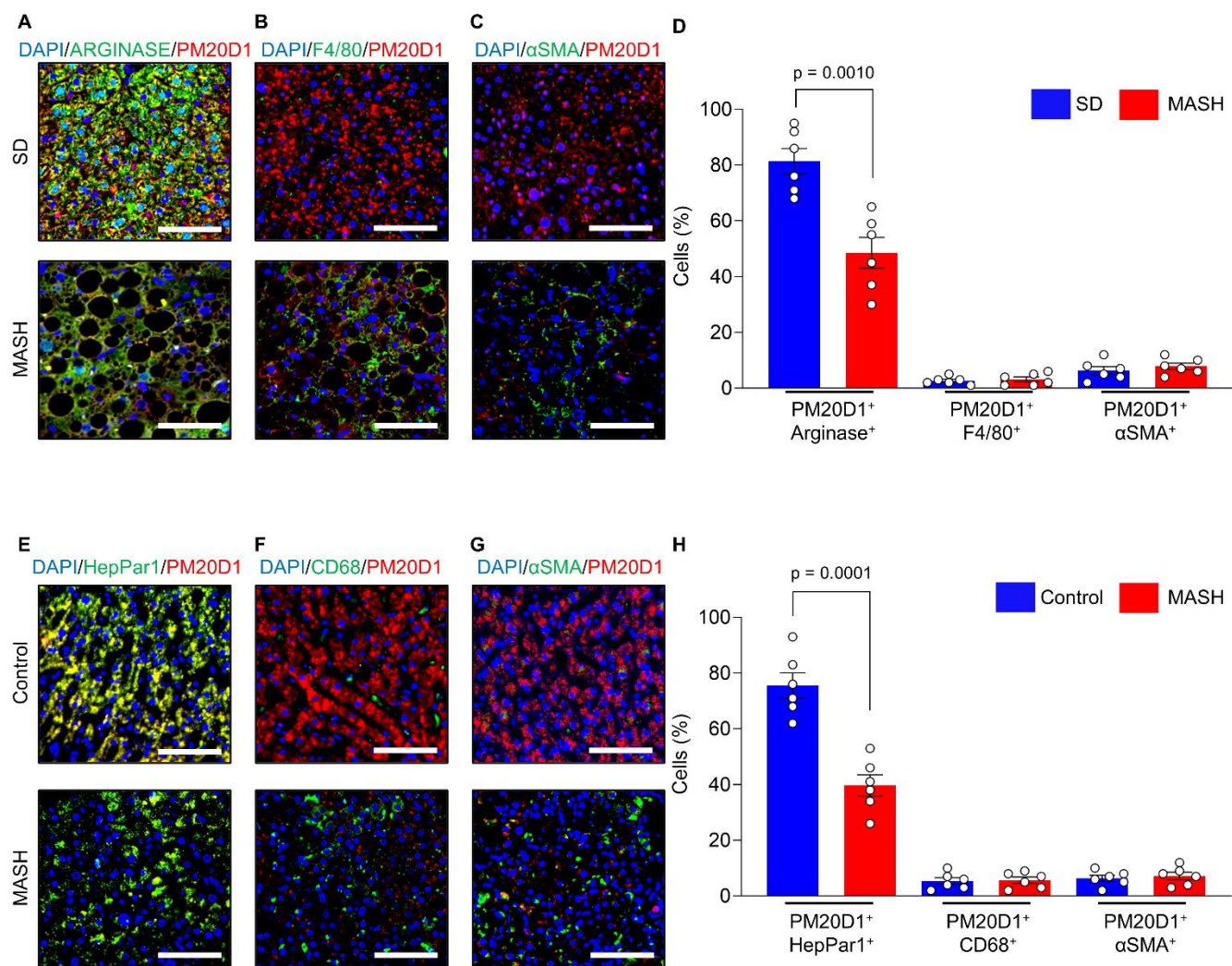
¹⁸ Department of Pharmaceutical Sciences and Department of Pharmacology, Wayne State University, Detroit, MI, USA

¹⁹ These authors contributed equally

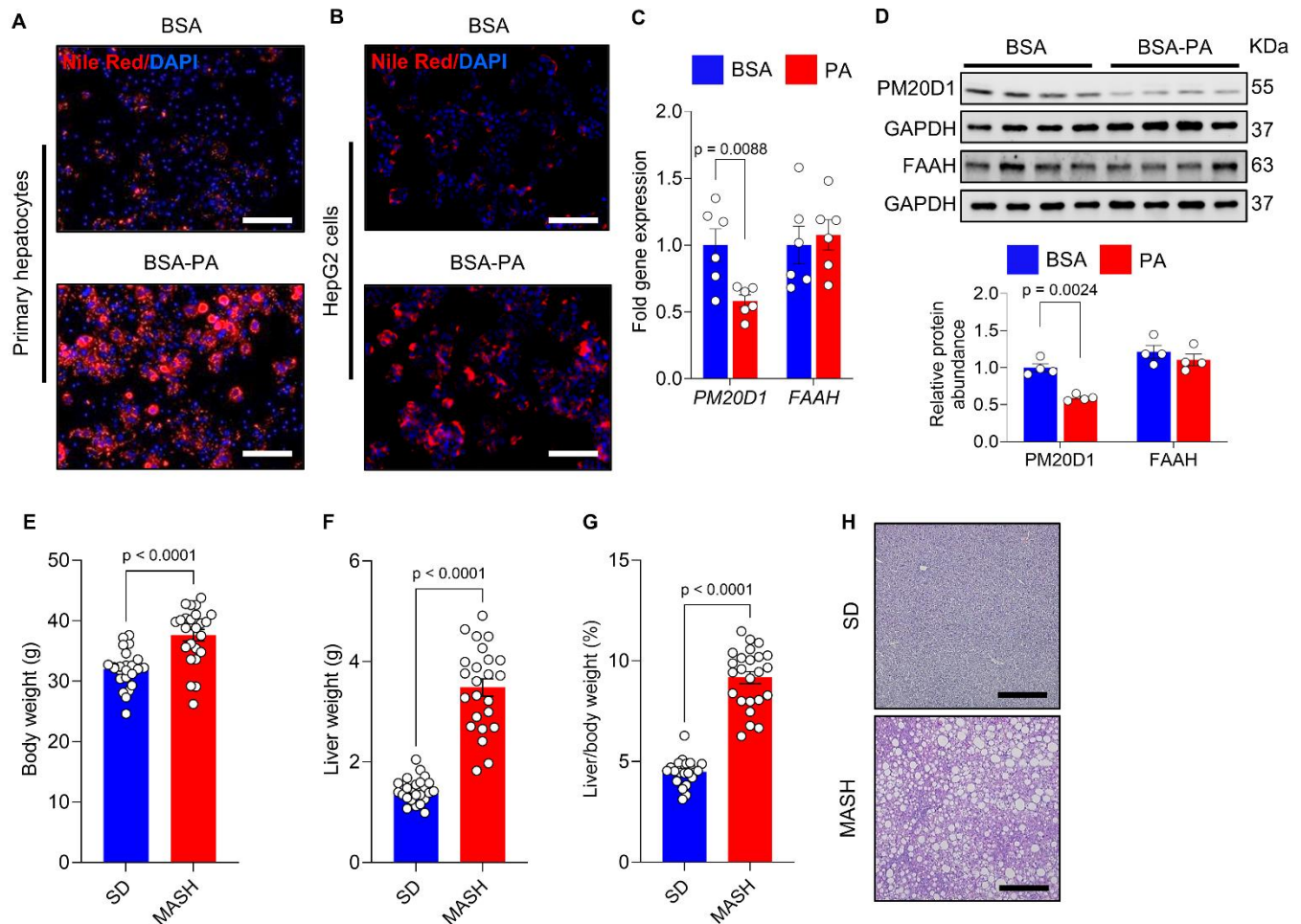
²⁰ Correspondence: oren.rom@lsuhs.edu



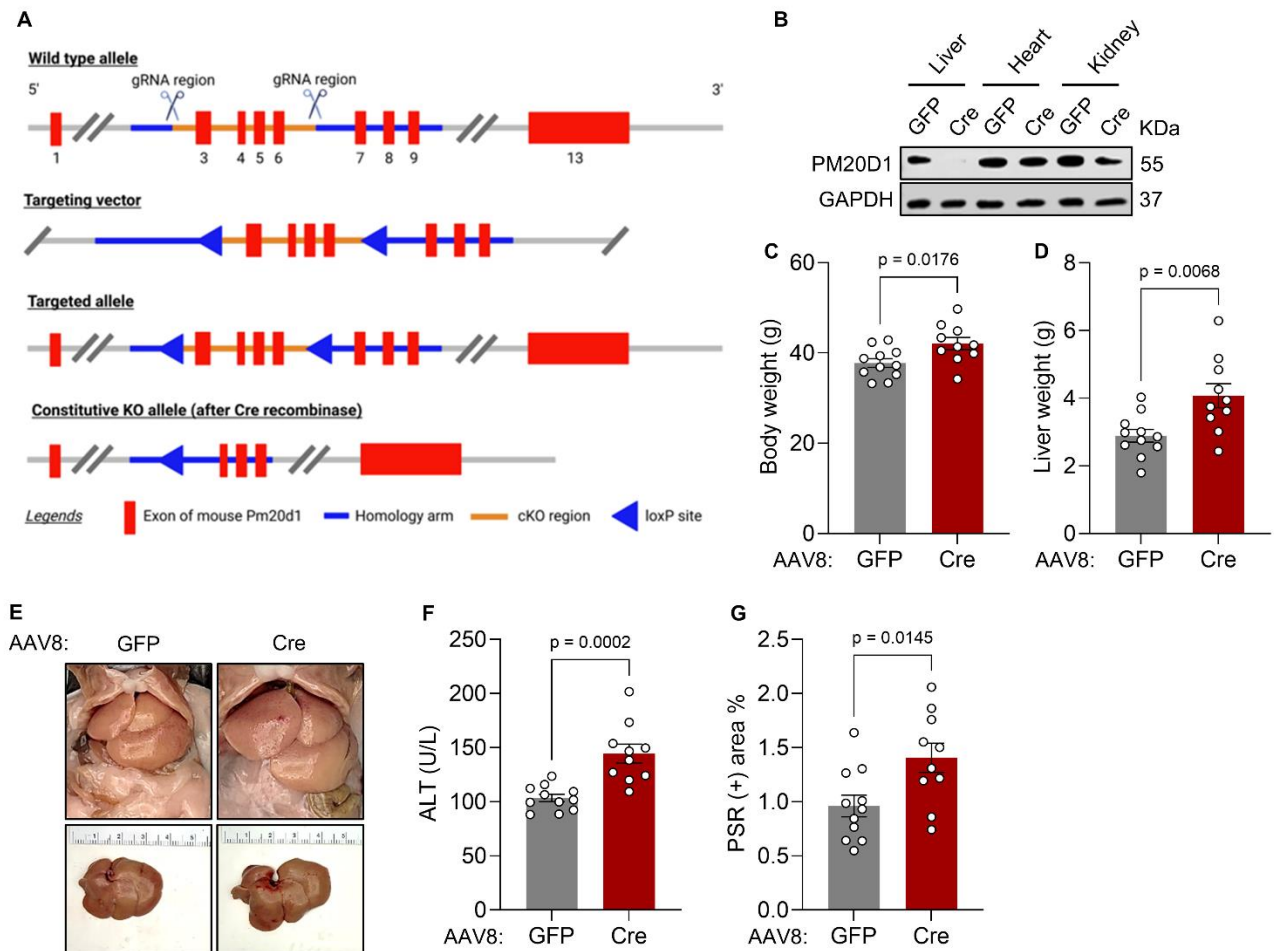
Supplemental Figure 1: Hepatic C18:1-Leu biosynthesis is impaired in humans and mice with MASH. Histopathological scores for steatosis, lobular inflammation, hepatocellular ballooning and NAFLD Activity Score (NAS) in human cohorts representing (A) patients with MASH (n=21) compared with healthy controls (n=8), and (B) patients with early MASLD (n=19) compared with healthy controls (n=25). Hepatic levels of (C) C18:1-Gly, (D) C20:4-Gly, and (E) C18:1-Leu in livers from patients with MASLD (n=19) or healthy controls (n=25). (F-I) Primary hepatocytes isolated from C57BL/6J mice (n=6) and treated with vehicle (EtOH), C18:1 (10 μ M) or C18:1-Leu (10 μ M) for 3 h. Metabolite abundance and isotopic labeling were quantified by LC-HRMS. Area under the curve (AUC) values are shown for total and M+2 labeled glutamate (F,G) and aspartate (H,I). (J) H&E staining of liver sections from mice fed a SD (n=4) and the FAT-MASH diet (n=6) for 16 weeks (Scale bar = 200 μ m). (K) Immunoblotting analysis of PM20D1 in liver from mice fed a SD (n=4) and the FAT-MASH diet (n=6) for 16 weeks. (L) H&E staining of liver sections from mice fed a SD (n=6) and the FPC diet (n=6) for 16 weeks (Scale bar = 200 μ m). (M) Immunoblotting analysis of PM20D1 in liver from mice fed a SD (n=6) and the FPC diet (n=6) for 16 weeks. Data are presented as medians and frequency distributions (A, B) or mean $\pm$ SEM (C-I, K, M). Statistical comparisons were made using Mann-Whitney U test (A-E, M), one-way ANOVA (F, H-I), Kruskal-Wallis (G) or unpaired t-test (K). A p value < 0.05 was considered statistically significant.



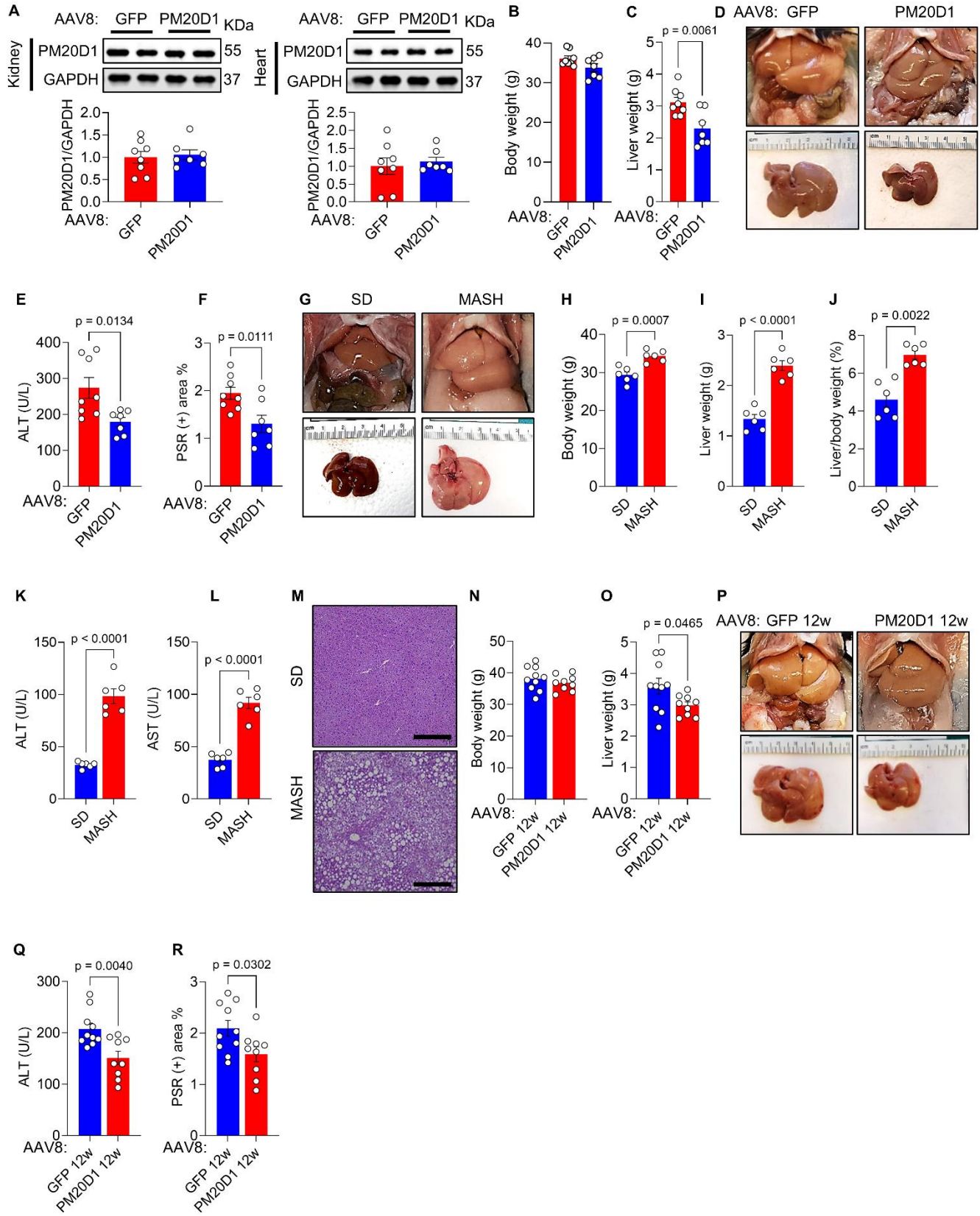
Supplemental Figure 2: PM20D1 is mainly expressed in hepatocytes and downregulated in MASH. Immunofluorescence analysis was performed on liver sections from C57BL/6J mice fed either a MASH-inducing diet or a standard diet (n=6). Images and quantification illustrate PM20D1 abundance, distribution and colocalization within distinct hepatic cell populations: (A) Arginase⁺, (B) F4/80⁺, and (C) αSMA⁺. (D) Quantification of PM20D1⁺ cells within each cell type. Immunofluorescence analysis of liver sections from patients with end-stage MASH (n=6) and healthy controls (n=6), illustrating PM20D1 colocalization with (E) HepPar1⁺, (F) CD68⁺, and (G) αSMA⁺. (Scale bars = 200 μm). (H) Quantification of PM20D1⁺ cells within each cell type. All data are expressed as mean ± SEM. Statistical comparisons were made using unpaired t-test (D, H). A p value < 0.05 was considered statistically significant.



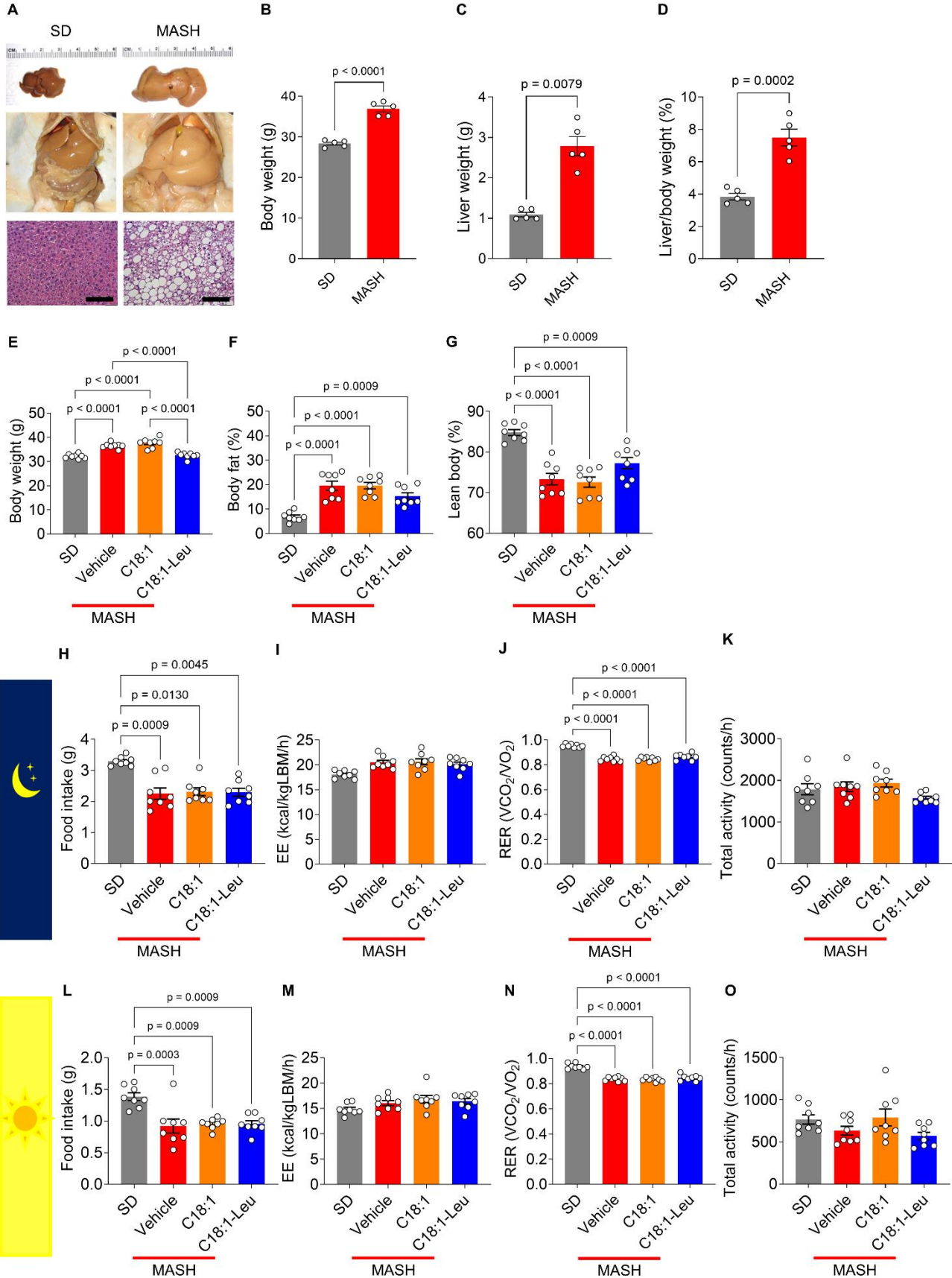
Supplemental Figure 3: Effect of palmitic acid on PM20D1, FAAH expression and metabolic flux analysis of C18:1-Leu in MASH. (A) Neutral lipids (red) stained with Nile Red, and nuclei (blue) labeled with DAPI in mouse primary hepatocytes treated with either BSA or BSA-palmitic acid (PA, 200 μ M) for 24 h (Scale bar = 200 μ m). (B) Neutral lipids (red) stained with Nile Red, and nuclei (blue) labeled with DAPI in HepG2 cells treated with either BSA (control) or palmitic acid (PA, 200 μ M) for 24h (Scale bar = 200 μ m). (C) qRT-PCR, (D) immunoblotting and densitometry analysis of PM20D1 normalized to GAPDH in HepG2 cells (n=4-6) treated either with BSA or BSA-PA (200 μ M) for 24h. (E-H) Male C57BL/6/J mice were fed a SD or MASH diet for 24 weeks. Before endpoint, the mice were either injected with $^{13}\text{C}_6$ -labeled leucine and sacrificed after 1, 2 and 6 h or sacrificed at endpoint (control, 0 h). (E) Body weight, (F) liver weight, and (G) liver/body weight ratio at endpoint. (H) H&E staining of liver sections (Scale bar = 200 μ m). All data are expressed as mean $\pm$ SEM. Statistical comparisons were made using unpaired t-test (C, D, F) or Mann-Whitney U-test (E, G). A p value < 0.05 was considered statistically significant.



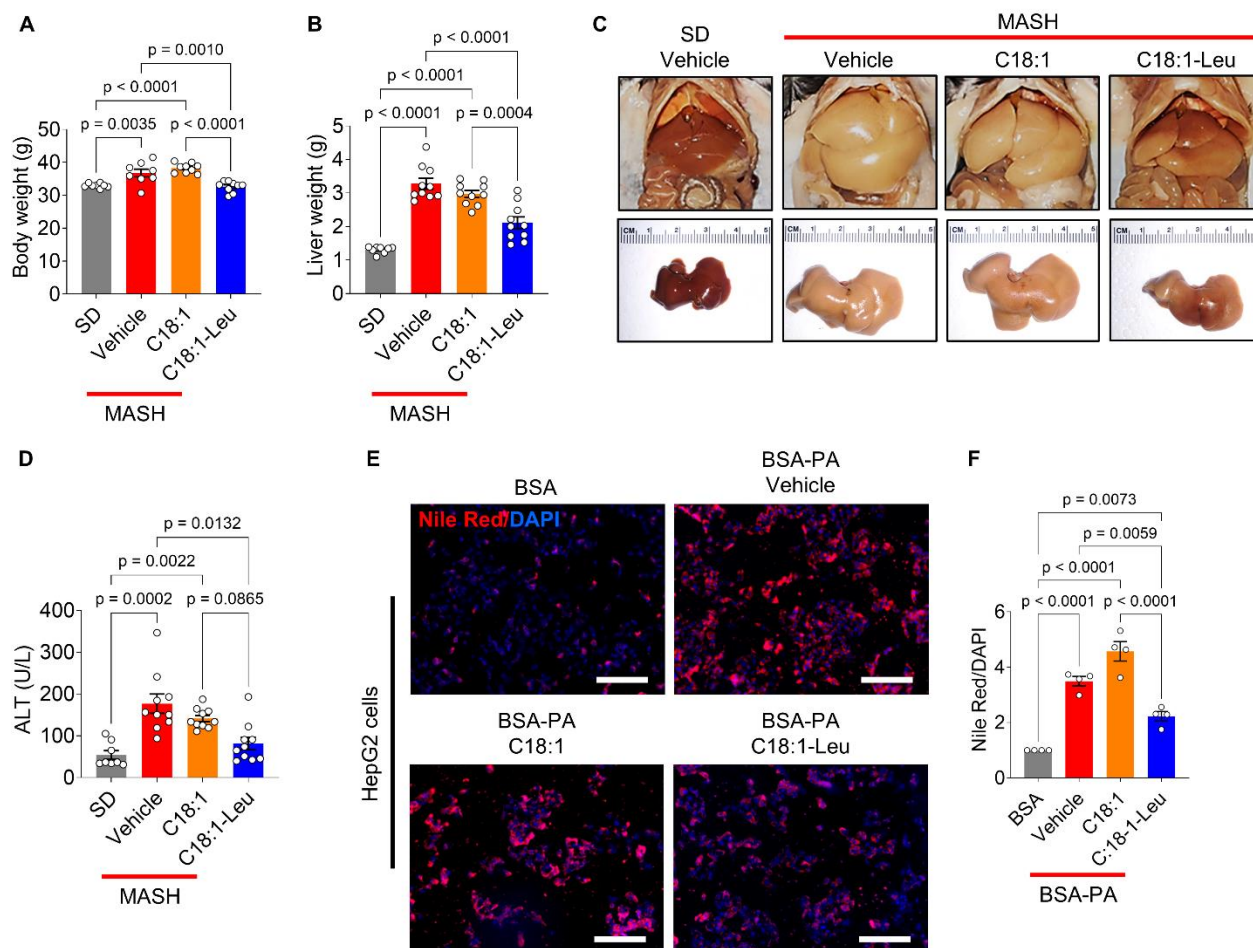
Supplemental Figure 4: Effect of hepatocyte specific ablation of PM20D1 on hepatic C18:1-Leu and onset of MASH. (A) Schema of the strategy employed for generating conditional *Pm20d1* knockout mice (*Pm20d1^{fl/fl}*) mice. (B-G) Male *Pm20d1^{fl/fl}* mice (8-week-old) were injected with AAV8-TBG-GFP (n=11) or AAV8-TBG-Cre (n=10) (2×10^{11} viral genomes per mouse) and fed the MASH diet for 12 weeks. (B) Immunoblotting analysis of PM20D1 in liver, kidneys and hearts. (C) Body weight, (D) liver weight, (E) representative images of the peritoneal cavity and liver, and (F) plasma ALT levels at endpoint. (G) PSR positive area (+) in liver sections. All data are expressed as mean $\pm$ SEM. Statistical comparisons were made using unpaired t-test (C, D, F and G). A p value < 0.05 was considered statistically significant.



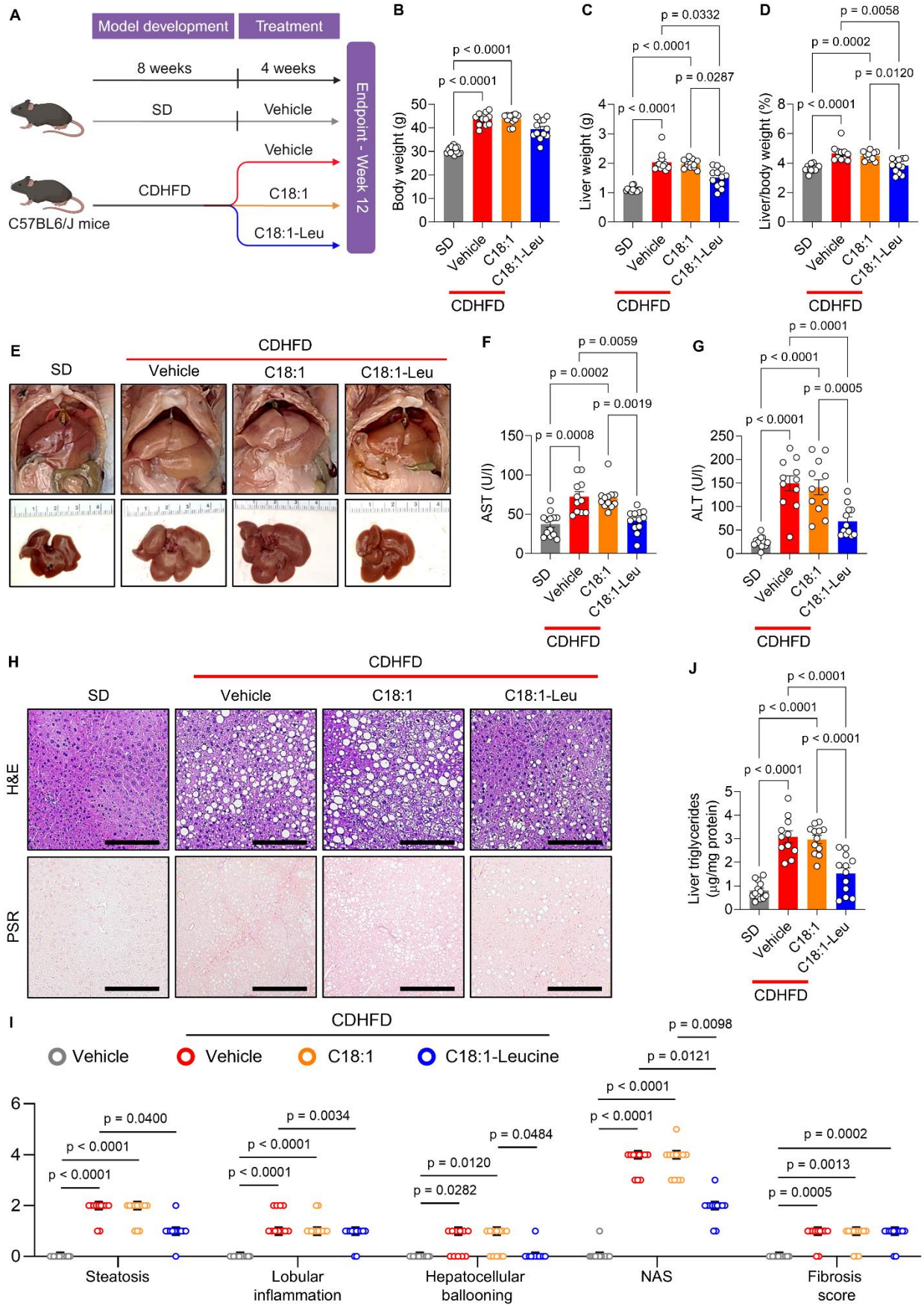
Supplemental Figure 5: PM20D1 overexpression restores hepatic C18:1-Leu levels, prevents MASH onset, and ameliorates established MASH. (A) Immunoblotting analysis of PM20D1 in kidneys and hearts from mice injected with AAV8-TBG-GFP (n=8) or AAV8-TBG-PM20D1 (n=7) and fed the MASH diet for 24 weeks at endpoint. (B) Body weight, (C) liver weight, (D) representative images of the peritoneal cavity and liver, and (E) plasma ALT levels at endpoint. (F) PSR positive area (+) in liver sections. (G) Representative images of the peritoneal cavity and liver of mice fed the SD or MASH diet for 12 weeks (n=6). (H) Body weight, (I) liver weight, (J) liver/body weight ratio, (K) plasma ALT levels, (L) plasma AST levels and (M) H&E staining of liver sections at week 12 (Scale bar = 100 μ m). (N) Body weight of mice injected with AAV8-TBG-GFP (n=10) and AAV8-TBG-PM20D1 (n=9) at endpoint. (O) Liver weight, (P) representative images of the peritoneal cavity and liver, (Q) plasma ALT levels, and (R) percentage of PSR positive area in liver sections at endpoint. All data are expressed as mean $\pm$ SEM. Statistical comparisons were made using unpaired t-test (A, C, E, F, H, I, K, L, N, O, Q and R) or Mann-Whitney U-test (A, B and J). A p value < 0.05 was considered statistically significant.



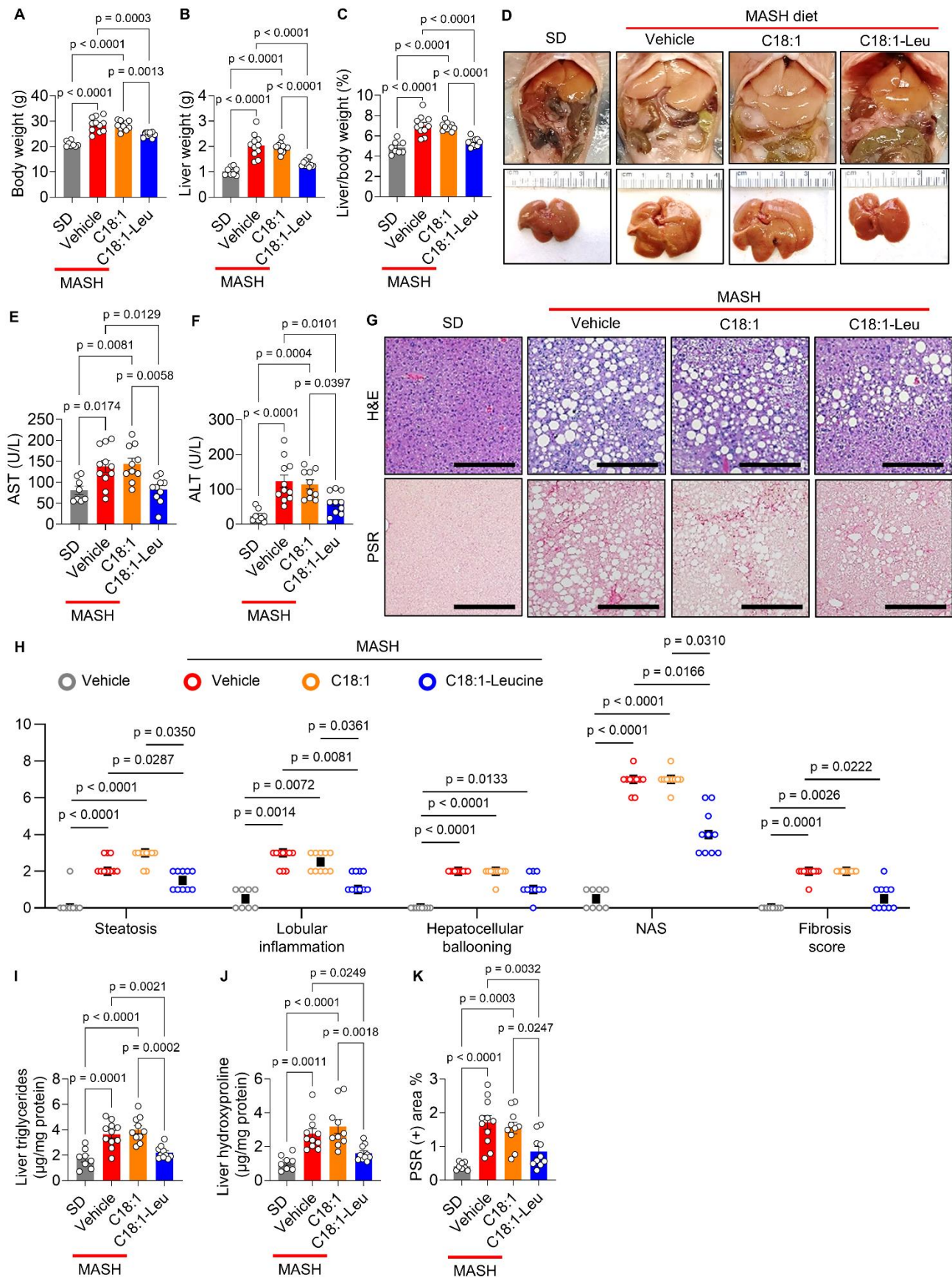
Supplemental Figure 6: Exogenous C18:1-Leu administration does not alter systemic energy metabolism in MASH. Male C57BL6/J mice (8-week-old) were fed a SD or MASH diet. After 16 weeks, mice were randomized to receive C18:1-Leu (I.P., 10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 6 weeks on the MASH diet. Mice fed the SD and given vehicle served as control. A subset of the mice (n=5) was sacrificed at week 16 prior to treatments to confirm MASH features. (A) Representative images of the peritoneal cavity and liver as well as H&E staining of liver sections from mice fed a SD or MASH diet for 16 weeks (n=5) (Scale bar = 100 μ m). (B) Body weight at week 16. (C) Liver weight at week 16. (D) Liver/body weight ratios at week 16. (E-O) Comprehensive lab animal monitoring system (CLAMS) combined with nuclear magnetic resonance (NMR)-based body composition analysis before endpoint (n=8). (E) Body weight before endpoint. (F) Body fat percentage before endpoint. (G) Lean body mass percentage before endpoint. (H-K) CLAMS analysis of (H) food intake, (I) energy expenditure (EE), (J) respiratory exchange ratio (RER), and (K) total activity during dark cycles. (L-O) CLAMS analysis of (L) food intake, (M) EE, (N) RER, and (O) total activity during light cycles. All data are expressed as mean $\pm$ SEM. Statistical comparisons were made using unpaired t-test (B, D), Mann-Whitney U-test (C), one-way ANOVA with Tukey's multiple comparisons test (E-G, I-O) or Kruskal-Wallis (H). A p value < 0.05 was considered statistically significant.



Supplemental Figure 7: C18:1-Leu ameliorates established MASH in male mice. Male C57BL6/J mice (8-week-old) were fed a SD or MASH diet. After 16 weeks, mice were treated with C18:1-Leu (10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 6 weeks on the MASH diet. Mice fed the SD and given vehicle served as control. (A) Body weight, and (B) liver weight at endpoint (n=8-10). (C) Representative images of the peritoneal cavity and liver at endpoint. (D) Plasma ALT levels at endpoint (n=8-10). (E) Neutral lipids were visualized with Nile Red stain (red), nuclei were labelled with DAPI (blue), and (F) quantified in HepG2 cells treated with C18:1-Leu or C18:1 (10 μ M) in the presence or absence of BSA-PA (200 μ M) for 24h (n=4). All data are expressed as mean $\pm$ SEM. Statistical comparisons were made using one-way ANOVA with Tukey's multiple comparisons test (A, B and F) or Kruskal-Wallis (D). A p value < 0.05 was considered statistically significant.

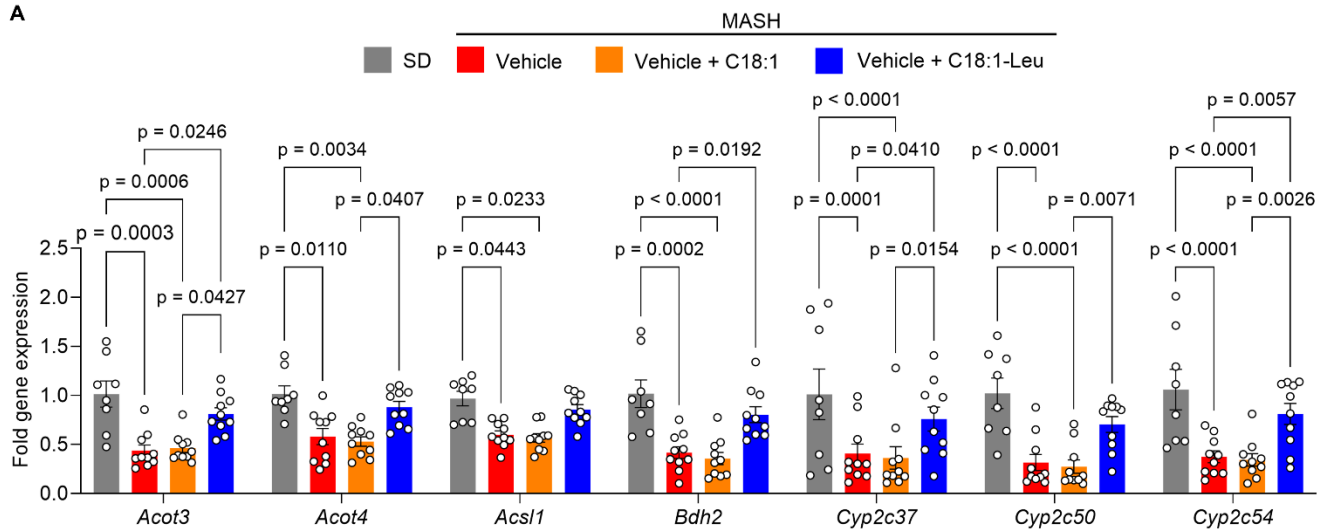


Supplemental Figure 8: Effect of C18:1-Leu treatment on mice fed choline-deficient high-fat diet (CDHFD). Male C57BL6/J mice (8-week-old) were fed a SD or CDHFD diet. After 8 weeks, mice were treated with C18:1-Leu (10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 4 weeks on the CDHFD diet (n=11-12). Mice fed the SD and given vehicle served as control (n=12). (A) Schema of the experimental design. (B) Body weight, (C) liver weight, and (D) liver/body weight ratio at endpoint. (E) Representative images of the peritoneal cavity and liver at endpoint. (F) Plasma AST and (G) Plasma ALT levels at endpoint. (H) H&E and PSR staining of liver sections (Scale bar = 200 μ m). (I) Histopathological analysis of steatosis, lobular inflammation, hepatocellular ballooning, NAS and fibrosis scores. (J) Liver triglyceride content normalized to protein concentration at endpoint. Data are presented as mean $\pm$ SEM or medians and frequency distributions (I). Statistical comparisons were made using Kruskal-Wallis with Dunn's multiple comparisons test (B-D, F and I) or one-way ANOVA with Tukey's multiple comparisons test (G, J). A p value < 0.05 was considered statistically significant, Scale bars, 200 μ m.

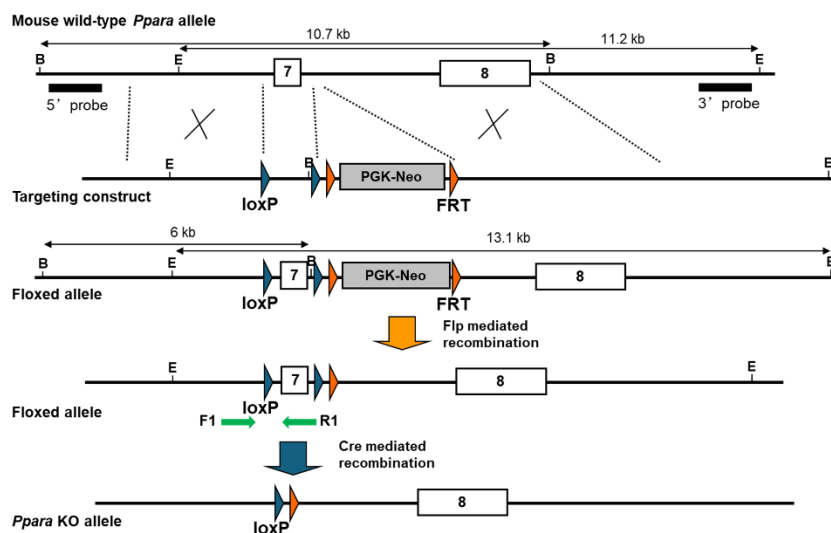


Supplemental Figure 9: C18:1-Leu ameliorates established MASH in female mice. Female C57BL6/J mice (8-week-old) were fed a SD or MASH diet. After 16 weeks, mice were treated with C18:1-Leu (10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 6 weeks on the MASH diet (n=10-11). Mice fed the SD and given vehicle served as control (n=8). (A) Body weight, (B) liver weight, and (C) liver/body weight ratio at endpoint. (D) Representative images of the peritoneal cavity and liver. (E) Plasma AST and (F) plasma ALT levels at endpoint. (G) H&E and PSR staining of liver sections (Scale bar = 200 μ m). (H) Histopathological analysis of steatosis, lobular inflammation, hepatocellular ballooning, NAS and fibrosis scores. (I) Liver triglyceride and (J) liver hydroxyproline content normalized to protein concentration. (K) Percentage of PSR positive area in liver sections. Data are presented as mean $\pm$ SEM or medians and frequency distributions (H). Statistical comparisons were made using one-way ANOVA with Tukey's multiple comparisons test (A, B, C, E, F, I, J, and K), and Kruskal-Wallis with Dunn's multiple comparisons test (H). A p value < 0.05 was considered statistically significant. (Scale bars = 200 μ m).

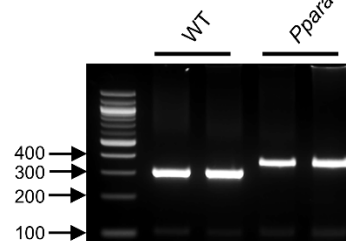
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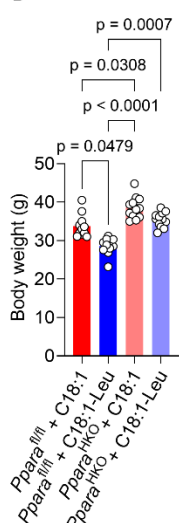
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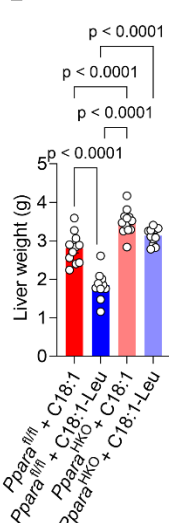
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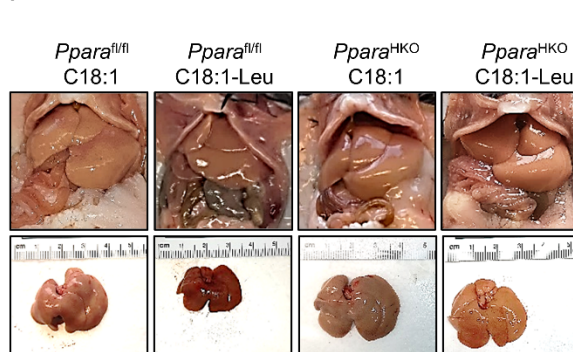
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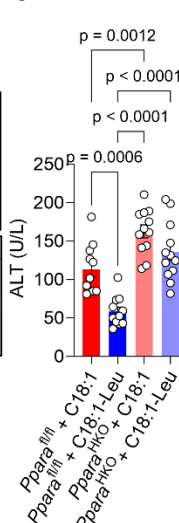
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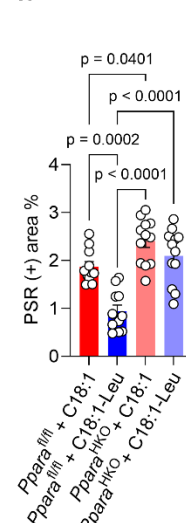
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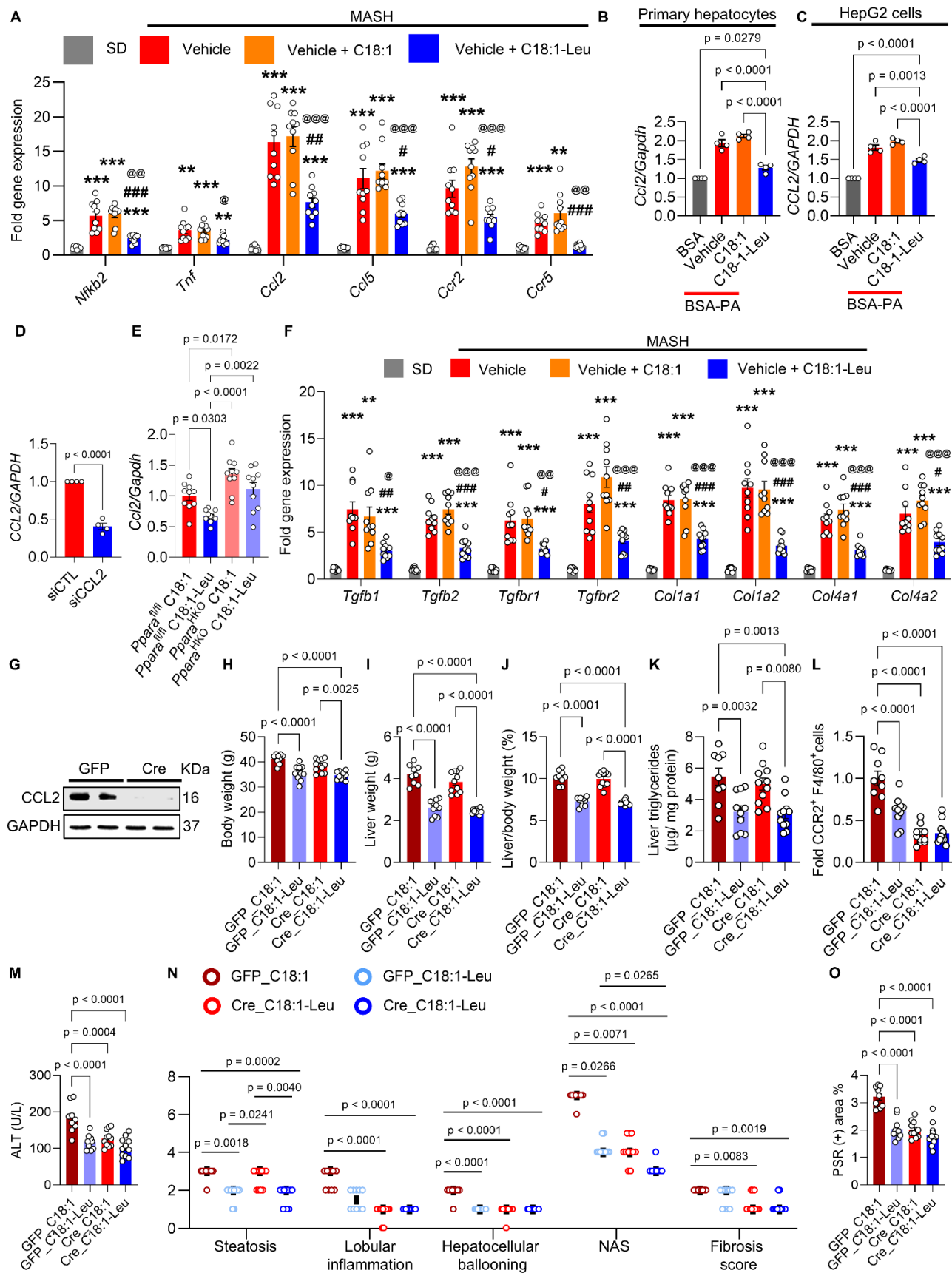
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H



Supplemental Figure 10: C18:1-Leu upregulates FAO genes and ameliorates MASH via hepatocyte-specific PPAR α . (A) Male C57BL6/J mice (8-week-old) were fed a SD or MASH diet. After 16 weeks, mice were treated with C18:1-Leu (10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 6 weeks on the MASH diet. Mice fed the SD and given vehicle served as control. qRT-PCR analysis of *Acot3*, *Acot4*, *Acs1*, *Bdh2*, *Cyp2c37*, *Cyp2c50*, and *Cyp2c54* normalized to *Gapdh* in livers at endpoint. (B) Schematic representation of the strategy employed for generating conditional *Ppara* knockout mice (*Ppara*^{fl/fl}) mice. A targeting vector containing exon 7 flanked by loxP sites, upstream and downstream homologous sequences, and a PGK-neo selection cassette was electroporated into mouse embryonic stem (ES) cells. Correctly targeted ES clones were microinjected into blastocysts and implanted into pseudopregnant females to generate chimeric F0 mice. Chimeric F0 mice were bred with C57BL/6J mice to achieve germline transmission of the floxed allele. Offspring carrying the floxed *Ppara* allele (F1 generation) were identified through PCR-based genotyping. The PGK-neo cassette was excised by crossing with FlpO recombinase-expressing mice. (C) PCR-based genotyping to distinguish wildtype (309 bp) from floxed (354 bp) alleles. (D-H) Male *Ppara*^{fl/fl} and *Ppara*^{HKO} mice (8-week-old) were fed the MASH diet. After 16 weeks, the mice were treated with C18:1-Leu or C18:1 (10 mg/kg/day) for an additional 6 weeks on the MASH diet (n=11-12). (D) Body weight, and (E) liver weight at endpoint. (F) Representative images of the peritoneal cavity and liver at endpoint. (G) Plasma ALT levels at endpoint. (H) Percentage of PSR positive area in liver sections at endpoint. All data are expressed as mean $\pm$ SEM. Statistical comparisons were made using two way ANOVA (A), one-way ANOVA with Tukey's multiple comparisons test (E, G, and H) or Kruskal-Wallis with Dunn's multiple comparisons test (D). A p value < 0.05 was considered statistically significant.



Supplemental Figure 11: C18:1-Leu blunts hepatic inflammation and fibrosis via a PPAR α -CCL2 negative crosstalk. (A) Male C57BL6/J mice (8-week-old) were fed a SD or MASH diet. After 16 weeks, mice were treated with C18:1-Leu (10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 6 weeks on the MASH diet. Mice fed the SD and given vehicle served as control. qRT-PCR of *Nfkb2*, *Tnf*, *Ccl2*, *Ccl5*, *Ccr2*, *Ccr5* normalized to *Gapdh* in the livers (n=8-10). **p<0.01, ***p<0.001 versus vehicle on SD; #p<0.05, ##p<0.01, ###p<0.001 versus vehicle on MASH diet; @@p<0.01, @@@p<0.001 versus C18:1 on MASH diet. qRT-PCR of *CCL2* normalized to *GAPDH* in (B) primary mouse hepatocytes and (C) HepG2 cells treated with C18:1-Leu or C18:1 (10 μ M) in the presence or absence of BSA-PA (200 μ M) for 24 h (n=4). (D) qRT-PCR of *CCL2* normalized to *GAPDH* in HepG2 cells transfected with siRNA against *CCL2* (siCCL2) or scrambled siRNA control (siCTL) for 24 h. (E) Male *Papra*^{f/f} and *Papra*^{HKO} mice (8-week-old) were fed the MASH diet. After 16 weeks, the mice were treated with C18:1-Leu or C18:1 (10 mg/kg/day) for an additional 6 weeks on the MASH diet (n=10). qRT-PCR of *Ccl2* normalized to *Gapdh* in the livers (n=11-12). (F) qRT-PCR of *Tgfb1*, *Tgfb2*, *Tgfbr1*, *Tgfbr2*, *Col1a1*, *Col1a2*, *Col4a1*, *Col4a2* normalized to *Gapdh* in the livers of mice described in (A) (n=8-10). ***p<0.001 versus vehicle on SD; ##p<0.01, ###p<0.001 versus vehicle on MASH diet; @@p<0.01, @@@p<0.001 versus C18:1 on MASH diet. (G) Immunoblotting of CCL2 normalized to GAPDH in primary mouse hepatocytes isolated from *Ccl2*^{f/f} mice injected with AAV8-TBG-GFP or AAV8-TBG-CRE (2 $\times$ 10¹¹ viral genomes per mouse). (H-O) *Ccl2*^{f/f} mice were injected with AAV8-TBG-GFP or AAV8-TBG-Cre (2 $\times$ 10¹¹ viral genomes per mouse) and placed on MASH diet for 16 weeks. After 16 weeks, mice were treated with C18:1-Leu or C18:1 (10 mg/kg/day) for an additional 6 weeks on the MASH diet (n=9-11). (H) Body weight, (I) liver weight, and (J) Liver/body weight ratios at endpoint (n=9-11). (K) Liver triglyceride content normalized to protein concentration at endpoint (n=9-11). (L) Liver sections were stained for F4/80 (red), CCR2+ (green) and DAPI (blue), analyzed for CCR2⁺F4/80⁺ cells and expressed as fold change (n=9-11). (M) Plasma ALT levels (n=9-11). (N) Histopathological analysis of steatosis, lobular inflammation, hepatocellular ballooning, NAS and fibrosis scores (n=9-11). (O) Percentage of PSR positive area in liver sections at endpoint (n=9-11). Data are presented as mean $\pm$ SEM or medians and frequency distributions (N). Statistical comparisons were made using two-way ANOVA (A, F), one-way ANOVA with Tukey's multiple comparisons test (B, C, E, H-M, O), Kruskal-Wallis with Dunn's multiple comparisons test (N), or unpaired t-test (D). A p value < 0.05 was considered statistically significant.

Supplemental Table 1: Spearman's correlation analysis of hepatic C18:1-Leu with steatosis, lobular inflammation, hepatocellular ballooning, NAS and fibrosis scores in mice and humans.

	Correlation with hepatic C18:1-Leu			
	Mouse		Human	
	<i>r</i>	P value	<i>r</i>	P value
Steatosis	-0.7939	0.0006	-0.5075	0.0050
Lobular inflammation	-0.7076	0.0032	-0.3219	0.0886
Hepatocellular ballooning	-0.6978	0.0039	-0.5340	0.0028
NAS	-0.6826	0.0050	-0.4270	0.0209
Fibrosis score	-0.7216	0.0023	-0.5280	0.0032

Values are presented as Spearman's correlation coefficients (*r*) and corresponding P values. Mouse data (n=16) were obtained from mice fed a standard diet or MASH-inducing diet for 24 weeks. Human data (n=29) were obtained from patients with end-stage MASH and healthy donors. NAS: NAFLD Activity Score.

Supplemental Table 2: Spearman's correlation analysis of hepatic PM20D1 protein abundance with steatosis, lobular inflammation, hepatocellular ballooning, NAS and fibrosis scores in mice and humans.

	Correlation with hepatic PM20D1 protein levels			
	Mouse		Human	
	<i>r</i>	P value	<i>r</i>	P value
Steatosis	-0.7559	0.0014	-0.3944	0.0343
Lobular inflammation	-0.7076	0.0032	-0.2832	0.1366
Ballooning	-0.7885	0.0007	-0.5031	0.0054
NAS	-0.5138	0.0437	-0.3678	0.0496
Fibrosis score	-0.7341	0.0048	-0.5365	0.0027

Values are presented as Spearman's correlation coefficients (*r*) and corresponding P values. Mouse data (n=16) were obtained from mice fed a standard diet or MASH-inducing diet for 24 weeks. Human data (n=29) were obtained from patients with end-stage MASH and healthy donors. NAS: NAFLD Activity Score.

Supplemental Table 3: Demographic and clinical characteristics of the study population and stratification by advanced MASH and healthy control groups.

	Total (n=29)	Control (n=8)	MASH (n=21)	p-value
Demographics				
Age (years, mean $\pm$ SEM)	62.3 $\pm$ 1.2	60.6 $\pm$ 2.4	63.0 $\pm$ 1.4	0.3946
Sex (% female/male)	48.2	62.5	42.9	0.4270
BMI (kg/m ²)	29.5 $\pm$ 1.2	26.3 $\pm$ 2.1	30.7 $\pm$ 1.3	0.3242
Race (C/AA/A/H)	25/2/1/1	5/1/1/1	20/1/0/0	0.0907
Cardiometabolic comorbidities				
Type 2 diabetes (%)	37.9	0.0	52.4	0.0116
Obesity (%)	44.8	37.5	47.6	0.6968

Data are presented as mean $\pm$ SEM for normally distributed continuous variables, and n (%) for categorical variables. Abbreviations: Caucasian (C), African American (AA), Asian (A), Hispanic (H). Normality was assessed using the Shapiro-Wilk test. Comparisons between groups were performed using Student's t-test for normally distributed continuous variables, Mann-Whitney U test non-normally distributed variables, and Chi-square test for categorical variables, as appropriate. P values are provided for descriptive purposes. Statistical significance was defined as $p < 0.05$.

Supplemental Table 4: Demographic characteristics of the study population and stratification by early MASLD and healthy control groups.

	Total (n=44)	Healthy (n=25)	MASLD (n=19)	p-value
Demographics				
Age (years, mean $\pm$ SEM)	51.1 $\pm$ 2.2	50.0 $\pm$ 3.1	52.6 $\pm$ 3.1	0.5709
Sex (% female/male)	40.9	48.0	31.6	0.3586
Race (C/AA/A/H)	44/0/0/0	25/0/0/0	19/0/0/0	>0.9999

Data are presented as mean $\pm$ SEM for normally distributed continuous variables, and n (%) for categorical variables. Abbreviations: Caucasian (C), African American (AA), Asian (A), Hispanic (H). Normality was assessed using the Shapiro-Wilk test. Comparisons between groups were performed using Student's t-test for normally distributed continuous variables, Mann-Whitney U test non-normally distributed variables, and Fisher's exact test for categorical variables, as appropriate. P values are provided for descriptive purposes. Statistical significance was defined as $p < 0.05$.

Supplemental Table 5: Human primers used for qRT-PCR analyses

Gene name (Human)	Forward primer	Reverse primer
Peptidase M20 domain containing 1 (<i>PM20D1</i>)	AGAAGCCCATCGCCTTGATTGC	CTCCTTTGGAGGAGCTGAAGAG
Fatty acid amide hydrolase (<i>FAAH</i>)	GGGGACCTGGTCTCAATTCTG	CAATCACGGTTTTGCGGTACA
C-C motif chemokine ligand 2 (<i>CCL2</i>)	GCTCAGCCAGATGCAATCA	AGATCTCCTTGGCCACAATG
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Supplemental Table 6: Mouse primers used for qRT-PCR analyses

Gene Name (Mouse)	Forward Primer	Reverse Primer
Peptidase M20 domain containing 1 (<i>Pm20d1</i>)	CAAAGTATAGCCACCTGTTACACC	GATCTTTTGGGGCTGTAGTTTCT
Fatty acid amide hydrolase (<i>Faah</i>)	AGCCAAATAAGTCTCCTCCAGTC	ATAAACCTTACAGAGCCCAGACC
Carnitine palmitoyltransferase 1A (<i>Cpt1a</i>)	AGATCAATCGGACCCTAGACAC	CAGCGAGTAGCGCATAGTCA
Acyl-CoA dehydrogenase medium chain (<i>Acadm</i>)	CTGTGATTCTTGCTGGAAATGA	GCCGTTGATAACATACTCGTCA
Acyl-CoA dehydrogenase long chain (<i>Acadl</i>)	CTATATTGCGAATTACGGCACA	ACACCTTGCTTCCATTGAGAAT
Acyl-CoA dehydrogenase very long chain (<i>Acadvl</i>)	GCAGATGAGTGCATCCAAATAA	TGAGTTCCTTTCTTTGTCCAT
Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha (<i>Hadha</i>)	ACCTCGGTGTAAAGCACAAAGT	GAGGTTTTGTCAGTGGTGATGA
Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit betta (<i>Hadhb</i>)	AAACAAGCAATGTGGCTAGAGAG	CAGACATTAACCAACACCACCA
Enoyl-CoA delta isomerase 1 (<i>Eci1</i>)	TGCTGTGACTACAGGGTTATGG	GATCCTCAGGTACCACCTCATC
Enoyl-CoA delta isomerase 2 (<i>Eci2</i>)	ACTACTGCAGTGGGAATGACCT	ATAGTCCCAGAAGGGTGACAGA
Acyl-CoA thioesterase 3 (<i>Acot3</i>)	ACTACGAGGACCTCCCTAAGGA	CATGGCAAAGCCAAGTTCACCC
Acyl-CoA thioesterase 4 (<i>Acot4</i>)	CATGGCTTTGCCACATTGGCTC	GGAAAGACCCAGAAGCCCAATG
Acyl-CoA synthetase long chain family member 1 (<i>Acs1</i>)	GAGCAATGATCACTCACCAAAA	TCTTAGCTCCATGACACAGCAT
3-hydroxybutyrate dehydrogenase 2 (<i>Bdh2</i>)	CGACTGGACGGCAAAGTTATT	CCTGGAGTTTGGACTCGTTGA

Cytochrome P450 2C37 (<i>Cyp2c37</i>)	CACGAGGCGTTTCTCACTCA	AGGGCTGCTCAGAATCTTTGT
Cytochrome P450 2C50 (<i>Cyp2c50</i>)	GAAAACGGCAACTATCCAT	CTTATGAAATTATGAGCAGGC
Cytochrome P450 2C54 (<i>Cyp2c54</i>)	CATGCCCTATACAAATGC	GGCAGTGTGTTCAGTATGG
C-C motif chemokine ligand 2 (<i>Ccl2</i>)	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
C-C motif chemokine ligand 5 (<i>Ccl5</i>)	ATATGGCTCGGACACCACTC	CCACTTCTTCTCTGGGTTGG
C-C motif chemokine receptor 2 (<i>Ccr2</i>)	GATGATGGTGAGCCTTGTCATA	AGTGAGCCCAGAATGGTAATGT
C-C motif chemokine receptor 2 (<i>Ccr5</i>)	GGATCAGGCTCAAGATGACC	ACACTCAGTATCATTTCTGG
Nuclear factor of kappa light polypeptide gene enhancer in B cells 2 (<i>Nfkb2</i>)	GAGGTTTCGGTTCTATGAGGATG	CTCTGCACTTCCTCCTTGTCTT
Tumor necrosis factor (<i>Tnf</i>)	CTGTGAAGGGAATGGGTGTT	GGTCACTGTCCCAGCATCTT
Transforming growth factor beta 1 (<i>Tgfb1</i>)	TGCGCTTGCAGAGATTAAAA	CTGCCGTACAACTCCAGTGA
Transforming growth factor beta 2 (<i>Tgfb2</i>)	CTAATGTTGTTGCCCTCCTACAG	GCACAGAAGTTAGCATTGTACCC
Transforming growth factor beta 1 (<i>Tgfb1</i>)	GGACCATTGTGTTACAAGAAAGC	CATGGCGTAACATTACAGTCTGA
Transforming growth factor beta 1 2 (<i>Tgfb2</i>)	TCCTAGTGAAGAACGACTTGACC	TACCAGAGCCATGGAGTAGACAT
Collagen, type I, alpha 1 (<i>Col1a1</i>)	TGAACGTGACCAAAAACCAA	GCAGAAAAGGCAGCATTAGG
Collagen, type I, alpha 2 (<i>Col1a2</i>)	AGGCAGGTCTGGGCTTTATT	CGTATCCACAAAGCTGAGCA
Collagen, type IV, alpha 1 (<i>Col4a1</i>)	CAGGCATAGTCAGACAACAGATG	TGGACAGCCAGTAAGAGTAGTCG
Collagen, type IV, alpha 2 (<i>Col4a2</i>)	CGGGTGTGAAAAGACCTATCGG	CTGGCATTCTCTGACGCCTTT
Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>)	CTGCGACTTCAACAGCAACT	GAGTTGGGATAGGGCCTCTC

Supplemental methods

Sex as a biological variable.

Sex was considered as a biological variable throughout this study. Human cohorts included both male and female participants. In animal studies, the majority of experiments were conducted in male mice; however, selected experiments were replicated in female mice to assess the generalizability of the findings across sexes. Sex-specific analyses and results are provided where applicable.

Animal studies

C57BL/6J (stock: 000664) were purchased from the Jackson Laboratory. Eight-week-old C57BL/6J male and female mice were fed ad libitum either a standard diet (SD LabDiet #5053, 13% of calories from fat) or an established (1-7) MASH-inducing diet (Research Diets #D17010103, 40% of calories from fat, 2% cholesterol) for 24 weeks prior to euthanasia and tissue harvest. Following 12 weeks on a MASH diet, mice were orally administered DT-109 (Diapin Therapeutics, Ann Arbor, MI, USA) by gavage at a dose of 500 mg/kg body weight/day or vehicle (H₂O, control) for an additional 12 weeks. Eight-week-old C57BL/6J male mice were fed for 16 weeks the SD, the fructose-palmitate-cholesterol (FPC) diet (3) (Teklad, TD.160785, 52% of calories from fat, 1.25% cholesterol) or the FAT-MASH diet (7) (Teklad diets, TD.120528, 42% of calories from fat, 1.25% cholesterol, and a high-sugar solution of 23.1 g/L d-fructose and 18.9 g/L d-glucose through the drinking water), with intraperitoneal injection of carbon tetrachloride at a dose of 0.32 mg/g body weight once per week.

Pm20d1 floxed mice (*Pm20d1^{fl/fl}*) were generated by Cyagen Biomodels LLC using CRISPR/Cas9-mediated genome editing by inserting LoxP sites flanking exons 3 and 6 of the *Pm20d1* gene on chromosome 1 (**Figure S4A**). To selectively induce Cre-mediated recombination in hepatocytes, 8-week-old male *Pm20d1^{fl/fl}* mice were administered 2×10^{11} viral genomes of AAV8-TBG-Cre (VectorBuilder, VB240129-1215pyw) by retro-orbital injection, whereas control mice received an equivalent dose of AAV8-TBG-GFP. Following viral delivery, mice were maintained on a MASH-inducing diet (Research Diets #D17010103) for 12 weeks. Genotyping was performed by PCR using the following primers: (Forward: 5'-ACGTAAACGGCCACAAGTTC-3'; Reverse: 5'-TGCTCAGAGGTAAACATGATCACT-3'). The wild-type and floxed alleles generated PCR products of 352 bp and 398 bp, respectively.

For PM20D1 overexpression studies, AAV8-PM20D1, encoding human PM20D1, and AAV8-GFP (control), both driven by the hepatocyte-specific TBG promoter (VectorBuilder), were administered via intraperitoneal injection into 8-week-old male C57BL/6J mice at a dose of 2×10^{11} viral genomes in a final volume of 200 μ L per mouse, as we previously described (4, 8). Injections were given either at the start of or after 12 weeks on a MASH diet (Research Diets #D17010103), continuing up to 24 weeks. For C18:1-Leu administration studies, eight-week-old male and female C57BL/6J mice were fed either the SD or the MASH diet (Research Diets #D17010103) for 16 weeks. Subsequently, the mice were randomized to receive daily intraperitoneal injections (I.P.) of C18:1-Leu (10 mg/kg/day), an equivalent amount of non-conjugated C18:1 or vehicle for an additional 6 weeks on the MASH diet. Mice fed the SD and given vehicle served as control.

Conditional *Ppara* knockout mice (*Ppara^{fl/fl}*) mice were generated on a C57BL/6J background by inserting loxP sites flanking exon 7 of the mouse *Ppara* gene. A targeting vector containing exon 7 flanked by loxP sites, upstream and downstream homologous sequences, and a PGK-neo selection cassette was electroporated into mouse embryonic stem (ES) cells. Correctly targeted ES clones were identified, microinjected into blastocysts, and implanted into pseudopregnant females to generate chimeric F0 mice. Chimeric F0 mice were bred with C57BL/6J mice to achieve germline transmission of the floxed allele. Offspring carrying the floxed *Ppara* allele (F1 generation) were identified through PCR-based genotyping. The PGK-neo cassette was excised by crossing with FlpO recombinase-expressing mice (The Jackson Laboratory, Strain #011065). Genotyping was performed using the following primers (Forward: 5'-

GGGTGCTCTGGCTGTGTTTATG-3'; Reverse: 5'-CAAGCTGGGTGGTAGGATGATTC-3') to distinguish wild-type (309 bp) from floxed (354 bp) alleles. *Ppara*^{fl/fl} mice were crossed with *Alb*-Cre mice (Jackson Laboratory, Strain #003574) to generate hepatocyte-specific *Ppara* knockout (*Ppara*^{HKO}) mice (**Figure S10B, C**). Specific hepatic deletion of PPAR α was confirmed by Western blot analysis (**Figure 7A**). *Ppara*^{fl/fl} and *Ppara*^{HKO} mice (8-week-old) were fed the MASH diet (Research Diets #D17010103). After 16 weeks, the mice were treated with C18:1-Leu or C18:1 (10 mg/kg/day) for an additional 6 weeks on the MASH diet. *Ccl2*^{fl/fl} mice (B6.Cg-*Ccl2*^{tm1.1Pame}/J, The Jackson Laboratory, Strain #: 016849) were injected with AAV8-TBG-GFP or AAV8-TBG-CRE (2×10^{11} viral genomes per mouse, VectorBuilder) and placed on the MASH diet. After 16 weeks, the mice were treated with C18:1-Leu or C18:1 (10 mg/kg/day) for an additional 6 weeks on the MASH diet.

Human studies

Liver specimens were obtained during orthotopic liver transplantation due to histologically confirmed end-stage MASH (n=21). Liver specimens were placed into formalin and further processed to formalin-fixed, paraffin-embedded tissue blocks or flash-frozen in liquid nitrogen and stored at -80 °C until analysis. Liver specimens from histologically confirmed healthy donors (control, n=8) were obtained from Xenotech. Liver specimens from donors with or without MASH were sectioned and stained with H&E and Picrosirius Red. Blinded assessment of NAS was performed by a gastrointestinal pathologist. Steatosis was scored from 0-3 (0, <5% steatosis; 1, 5-33%; 2, 34-66%; and 3, >67%). Hepatocyte ballooning was scored from 0-2 (0, normal hepatocytes; 1, normal-sized with pale cytoplasm; and 2, pale and enlarged hepatocytes, at least twofold). Lobular inflammation was scored from 0-3 based on foci of inflammation counted at 20 $\times$ (0, none; 1, <2 foci; 2, 2-4 foci; and 3, $\geq$ 4 foci). NAS was calculated as the sum of steatosis, hepatocyte ballooning and lobular inflammation scores **Supplemental Figure1A**. Fibrosis was scored 0-4 (0: no fibrosis; 1: perisinusoidal or portal fibrosis; 2: perisinusoidal and portal fibrosis; 3: bridging fibrosis; 4: cirrhosis) (4, 5, 9). Spearman's correlations between hepatic levels of C18:1-Gly, C20:4-Gly, C18:1-Leu and NAS and fibrosis scores were evaluated. Patient demographics are detailed in **Supplemental Table 3**.

To better capture early stages of disease progression, an independent sex- and age-matched human cohort was assembled. Deidentified human liver specimens from individuals with early-stage MASLD were collected as previously described (10, 11). Samples with a NAS score $\geq$ 4 (12, 13) were excluded, resulting in a cohort of 44 samples. MASLD was defined by $\geq$ 5% macrovesicular steatosis on histopathology, whereas healthy controls exhibited <5% steatosis. The MASLD group included 19 individuals (mean age 52.6 ± 3.1 years; 31% female) and had a median NAS score of 3 (interquartile range [IQR], 2-3). The healthy control group comprised 25 individuals (mean age 50.0 ± 3.2 years; 48% female) with a median NAS score of 0 (IQR, 0-0) **Supplemental Figure1B**. Patient demographics are detailed in **Supplemental Table 4**.

Luciferase assays

As we previously described (1), HepG2 cells (1×10^4 cells/well) obtained from ATCC (#HB-8065) were seeded in 96-well plates and transfected using Lipofectamine 3000 (Invitrogen, L3000-015) with the following plasmids per well: 80 ng PPReX3-TK-luciferase reporter (pGL3/PPReX3, Addgene), 10 ng human PPAR α expression vector (pcDNA3.1/hPPAR α , NM_001001930), and 10 ng *Renilla* luciferase control plasmid (pRL-TK, Promega). After 18 hours, cells were treated for 24 hours with either vehicle (EtOH), Wy-14643 (10 or 25 μ M), leucine (1 mM), glycine (1 mM), DT-109 (1 mM), C18:1 (10 or 25 μ M), or C18:1-Leu (10 or 25 μ M). Cells were then lysed, and luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, E1980) on a CLARIOstar Plus Multimode Plate Reader, following the manufacturer's instructions.

Histology, immunohistochemistry, and immunofluorescence

Histological procedures were conducted by technicians blinded to experimental groups at either the University of Michigan IVAC Histology Laboratory or Louisiana State University Health Shreveport, as we previously described (1-7). Briefly, Formalin-fixed liver tissues were sectioned at 4 μ m thickness using a M355S rotary microtome (ThermoFisher Scientific) and mounted on glass slides. Hematoxylin and eosin (H&E) staining was performed using standard protocols (ThermoFisher Scientific). For Picrosirius Red staining, slides were incubated with 0.2 N phosphomolybdic acid for 3 minutes, stained with 0.1% Sirius Red in saturated picric acid (Rowley Biochemical Inc.) for 90 minutes, then transferred to 0.01 N hydrochloric acid for 3 minutes. Picrosirius Red staining was used to score hepatic fibrosis from 0-4 (0: no fibrosis; 1: perisinusoidal or portal fibrosis; 2: perisinusoidal and portal fibrosis; 3: bridging fibrosis; 4: cirrhosis) (9). Immunofluorescence was performed using rabbit anti-PM20D1 (St John's Laboratory, STJ195978, 1:100), mouse anti-Arginase1-CoraLite® Plus 488 (Proteintech, CL488-66129, 1:200) rat anti-F4/80 (Bio-Rad ABD Serotec #MCA497R, 1:400), mouse anti-CD68 (Cell Signaling Technology, 41077S, 1:200), mouse anti- α SMA-Cy3 (Sigma Aldrich, C6198, 1:200), mouse anti-HepPar1 (Cell Signaling Technology, 96422S, 1:200), rabbit anti-CCR2 (Abcam #ab273050, 1:250), and nuclei were visualized with DAPI. To visualize F4/80, goat-anti-rat secondary antibody (Invitrogen #A-21247, 1:200), for Arginase-1, CD68, α SMA and HepPar1 donkey-anti-mouse secondary antibody was used (Invitrogen #A10036, 1:200), while PM20D1 and CCR2 were visualized using donkey-anti-rabbit secondary antibody (1:200, Invitrogen #A-21206). For Nile Red in hepatocytes, cells were fixed with 3.7% neutral buffered formalin and stained with Nile Red (TCI Chemicals #N0659, 1:2,000) and DAPI (MP Biomedicals #0215757410, 1:50,000) for 30 min. Liver sections and cells were imaged on a Keyence BZ-X810 all-in-one fluorescence microscope. Images were analyzed using the Keyence BZ-X800 analyzer software.

Analysis of hepatic NAA levels and biosynthesis by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

To assess C18:1-Gly, C20:4-Gly, and C18:1-Leu in liver samples, approximately 50 mg of liver tissue was homogenized using the Precellys Evolution homogenizer (Bertin Technologies, 6,500 rpm). To minimize matrix effect, liver homogenization was diluted 5 times in water, and 150 μ L of acetonitrile containing 25 nM internal standard was added to 30 μ L of the diluted liver homogenization, followed by vortexing for 10 min at room temperature. The mixture was centrifuged at 3,500 rpm for 10 min at 4°C, and the supernatant was transferred to autosampler vials for LC-MS/MS analysis. The method consisted of a Shimadzu LC-20AD HPLC system (Kyoto, Japan), and chromatographic separation of the three NAAs was achieved using a Waters XBridge reverse-phase C18 column (5 cm x 2.1 mm I.D., packed with 3.5 μ m) at 25°C. A total of 5 μ L of the supernatant was injected. The flow rate of the gradient elution was 0.4 mL/min with mobile phase A (0.1% formic acid in purified deionized water) and mobile phase B (0.1% formic acid in acetonitrile). An AB Sciex 4500 QTrap mass spectrometer equipped with an electrospray ionization source (ABI-Sciex, Toronto, Canada) in the positive-ion multiple reaction monitoring (MRM) mode was used for detection. Protonated molecular ions and its respective ion products were monitored at the transitions of m/z 396.3 > 132.1 for C18:1-Leu, 340.5 > 76.1 for C18:1-Gly, 362.5 > 287.0 for C20:4-Gly, and 455.2 > 425.2 for the internal standard. Mass spectrometry was adjusted to maximize analytical sensitivity and specificity of detection. Data were processed with software Analyst (version 1.6). The analytical curves for the quantification of three NAA standards were constructed using 10 nonzero standards by plotting the peak area ratio of the analyte to the internal standard versus the sample concentration, using water as the matrix. $^{13}\text{C}_6$ -labelled C18:1-Leu was quantified based on the analytical curve of C18:1-Leu. To prevent carryover, the concentration ranges evaluated were from 0.5 to 500 ng/mL. The linear regression analysis of three NAAs was performed by plotting the peak area ratio (y) against the NAA concentration (x2). The linearity of the relationship between peak area ratio and

concentration was demonstrated by the correlation coefficient ($r > 0.99$) obtained for the linear regression. To assess the rates of *de novo* C18:1-Leu biosynthesis, male C57BL6/J mice were fed a SD or MASH diet for 24 weeks. Before endpoint, the mice were either injected intraperitoneally with $^{13}\text{C}_6$ -labeled leucine (Cambridge Isotope Laboratories, 100 mg/kg) and sacrificed after 1, 2 and 6 h or sacrificed at endpoint (control, 0 h). Livers were processed as described above and assessed for $^{13}\text{C}_6$ labelled C18:1-Leu using LC-MS/MS.

Primary hepatocyte isolation and culture

Primary hepatocytes were isolated from 8-10-week-old mice maintained on a SD. Following euthanasia, the left lateral liver lobe was excised and rinsed with cold phosphate-buffered saline (PBS). Hepatocyte isolation was performed using the gentleMACS™ Perfuser system and associated reagents (Miltenyi Biotec), following the manufacturer's protocol. Briefly, the liver lobe was mounted on the grid within the perfusion chamber, and the lid was attached to the base to form a sealed chamber. Pre-warmed pre-digestion buffer was added and perfused through the tissue via an automated program comprising priming, washing, equilibration, and enzymatic digestion steps. A collagenase-containing enzymatic solution was used to degrade extracellular matrix components and facilitate cell dissociation. The digested liver tissue was transferred to a gentleMACS™ C Tube for mechanical dissociation, and the resulting cell suspension was filtered through a 70- μm MACS SmartStrainer to remove debris and non-parenchymal cells. Hepatocytes were pelleted by low-speed centrifugation ($50 \times g$, 3 min), resuspended in warm thawing/plating medium, and seeded at ~80% confluency onto 0.01% (w/v) collagen-coated plates (Advanced Biomatrix, NC0476635). After 3-4 hours, non-adherent cells were removed by gentle PBS wash. Cultures were maintained in William's E Medium (Gibco, A1217601) supplemented with hepatocyte maintenance additives (Gibco, CM4000) at 37°C in a 5% CO_2 humidified incubator.

Fatty acid β -oxidation analysis using ^{13}C isotope tracing and intracellular metabolite profiling by LC-MS/MS.

Isotope-labeled U- $^{13}\text{C}_{16}$ -palmitic acid (Cambridge Isotope Laboratories, CLM-409-0.1) was dissolved in 3.9 mL of 0.1 M NaOH by heating at 75 °C with intermittent vortexing until completely solubilized. Separately, a 10% fatty acid-free bovine serum albumin (BSA; Sigma, A4612) solution in distilled water was prepared and warmed to 55 °C. To prepare the BSA-labelled palmitate stock solution, 1 mL of the 100 mM sodium palmitate solution was added dropwise to 9 mL of the prewarmed 10% BSA solution under continuous stirring and incubated at 55°C for an additional 15 min to allow conjugation. The resulting 10 mM BSA-palmitate stock solution was sterile-filtered through a 0.22- μm membrane, aliquoted, and stored at -20°C until use.

Primary mouse hepatocytes were isolated as described above. Following overnight incubation, the culture medium was replaced with William's E Medium (Gibco, A1217601) supplemented with insulin transferrin selenium (ITS; Sigma, I3146-5ML) and the tracer BSA-labelled palmitate (100 μM). Cells were then treated with vehicle (EtOH), C18:1 (10 μM), or C18:1-Leu (10 μM) for 3h (14). After 3h, cells were washed twice with ice-cold PBS. Thereafter, intracellular metabolites were extracted with a cold solution of methanol, acetonitrile and water (5:3:2). The cell extracts were centrifuged at 16,000g for 10 min at 4 °C, and the supernatants were further subjected to LC-MS analysis.

For hydrophilic interaction liquid chromatography (HILIC) analysis, samples were injected by liquid chromatography (LC)-HRMS. LC mobile phase A (MPA) water containing 20 mM ammonium bicarbonate and 0.1% ammonium hydroxide (pH~9.5), and mobile phase B (MPB) was acetonitrile. Thermo Vanquish LC system included a Millipore SeQuant ZIC-pHILIC column (5 μm particle size, 150 x 4.6 mm) with column compartment kept at 30°C. The autosampler tray was chilled to 4°C. The mobile phase flow rate was 200 $\mu\text{L}/\text{min}$, and the gradient elution program was: 0-2 min, 85% MPB; 2-17 min, 85-20% MPB; 17-

19 min, 20% MPB; 19-21 min, 20-85% MPB. The total run time was 27 min. Data were acquired using a Thermo Orbitrap Exploris 240 Mass Spectrometer under ESI positive/negative ionization (polarity switching) mode at a resolution of 120,000. All the raw files were imported to Skyline-Daily and Thermo Scientific Compound Discoverer 3.5 software for final analysis. The relative abundance of each metabolite was normalized total peak intensity (15).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from ~50 mg of liver tissue using TRIzol reagent (Invitrogen, #15596018) and Precellys soft tissue homogenizing ceramic beads (Cayman Chemical, #10011152) in a Precellys Evolution homogenizer (Bertin Technologies). Phase separation was achieved by chloroform extraction, and RNA was purified from the aqueous phase using the RNeasy Mini Kit (Qiagen, #74106) according to the manufacturer's protocol. For cultured cells, total RNA was extracted directly using the RNeasy Mini Kit (Qiagen, #74106). cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, #18080-051) on a Mastercycler Nexus Gradient thermocycler (Eppendorf), following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, #1725271) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer sequences are listed in **Supplemental Tables 3 and 4** (Integrated DNA Technologies). Gene expression was normalized to GAPDH and analyzed using the $\Delta\Delta C_t$ method, with results expressed as fold change relative to control groups.

Protein isolation and immunoblotting

Protein was extracted from ~50 mg of tissue homogenized in RIPA lysis and extraction buffer (G Biosciences, #786-489) supplemented with 1% Halt Protease Inhibitor Cocktail (Thermo Scientific, #78429) and 1% Phosphatase Inhibitor Cocktail A (Alfa Aesar, #J65354.LQ), using Precellys soft tissue ceramic beads in a Precellys Evolution homogenizer. For cell lysates, proteins were extracted using either the RIPA buffer with inhibitors above or 2× Laemmli Sample Buffer (Bio-Rad, #1610737). Protein concentrations were quantified using the Quick Start Bradford 1× Dye Reagent (Bio-Rad, #5000205). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes, followed by immunoblotting with primary antibodies: anti-PM20D1 antibody (St John's Laboratory, STJ195978, 1:1000), anti-FAAH antibody (Proteintech, 17909-1-AP, 1:1000), anti-MCP-1/CCL2 antibody (Proteintech, 26161-1-AP, 1:1000), anti-PPAR alpha antibody (Abcam, ab126285, 1:1000), anti-GAPDH antibody (Proteintech, 60004-1-Ig, 1:10000) and secondary antibodies donkey anti-rabbit IgG (LI-COR Biosciences, #926-68073, 1:10000), donkey anti-Mouse IgG (LI-COR Biosciences, #926-32212, 1:10000), Peroxidase AffiniPure® Donkey Anti-Rabbit IgG (Jackson ImmunoResearch, #711-035-152, 1:10000), and Peroxidase AffiniPure™ Donkey Anti-Mouse IgG (Jackson ImmunoResearch, #715-035-150, 1:10000). Blots were visualized using the Li-Cor Odyssey XF Imaging System. Densitometry analysis was performed with Image Studio Lite v5.2, and protein expression levels were normalized to GAPDH as internal loading control.

Single nuclei RNA-seq analysis

The MASH snRNA-seq data were downloaded from GEO (Accession: GSE212837) and processed using the Seurat package (v5.0.1) in R (v4.3.2). Filtered data matrices were imported, and cells with mitochondrial mRNA percentages greater than 10% were removed. The filtered data were processed using the standard Seurat pipeline. Cells were annotated based on marker genes identified in the original paper (16) and MASH study (17). To compare *Pm20d1* expressions in MASH versus Chow hepatocytes, chow liver snRNA-seq data were downloaded from GEO (Accession: GSE212327) and integrated with

the MASH snRNA-seq data. *Pm20d1* expression data were visualized using violin plots generated with Seurat, and adjusted p-values were obtained using the FindMarkers function by comparing MASH and Chow hepatocytes.

Click-iT nascent RNA capture assay

The turnover of *PM20D1* mRNA was assessed using the Click-iT Nascent RNA Capture Kit (Life Technologies, C10365) according to the manufacturer's protocol, and as we previously described (4). HepG2 cells (3×10^6 per well in a 6-well plate) were treated with BSA or BSA-PA for 24 hours in the presence or absence of the alkyne-modified nucleoside EU (0.25 mM). After treatment, the medium was aspirated, and cells were washed twice to eliminate residual uridine analogue. Furthermore, the cells were lysed, and total mRNA was extracted. The Click-iT reactions were performed to conjugate biotinylated azide to EU-labeled mRNA, using 0.5 mM biotinylated azide per 1 mg of mRNA. Thereafter, the EU-labeled mRNAs were captured and precipitated utilizing streptavidin T1 magnetic beads, followed by thorough washing. Purified nascent RNA was subsequently used for cDNA synthesis and quantification of *PM20D1* expression by qRT-PCR.

Biochemical analysis of plasma and liver tissue

Following mouse euthanasia, plasma was separated from whole blood by centrifugation in PST tubes with lithium heparin (BD Microtainer #365985). AST and ALT were measured in mouse plasma using the EnzyChrom Aspartate Transaminase or Alanine Transaminase Assay Kits (BioAssay Systems #EASTR-100 and #EALT-100, respectively) as per the manufacturer's instructions. Hepatic lipids were extracted as we described previously (1-4, 18). Lipids were extracted using 3:2 hexane:isopropanol, and the aqueous components were separated out using PBS. The remaining hexane fraction was permitted to evaporate for 48 hours. Triglycerides were determined using the LabAssay Triglyceride measurement kit (FUJIFILM Wako #632-50991), according to the manufacturer's instructions, and normalized to protein concentrations. Liver hydroxyproline content was measured using the Abcam hydroxyproline assay kit (#ab222941) per the manufacturer's instructions and normalized to protein concentrations.

Mouse comprehensive laboratory animal monitoring system (CLAMS) and body composition analysis

As we previously described (2, 3), body composition including fat mass, lean mass, and free fluid was measured in conscious, unrestrained mice using a nuclear magnetic resonance (NMR)-based body composition analyzer (Minispec LF90II; Bruker Optics, Billerica, MA). The device was calibrated daily using a manufacturer-supplied standard to ensure accuracy and consistency. Oxygen consumption (VO_2), carbon dioxide production (VCO_2), and motor activity were assessed using the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments), an integrated open-circuit indirect calorimetry platform equipped with optical beam sensors for activity monitoring. Mice were weighed prior to each measurement session and individually housed in sealed metabolic chambers (7.9" $\times$ 4" $\times$ 5") with ad libitum access to food and water. Experiments were conducted in a temperature-controlled room maintained at 20-23°C with 12-12 hours (6:00PM~6:00AM) dark-light cycles. Metabolic parameters were continuously recorded over a 48-hour period. VO_2 and VCO_2 were sampled sequentially for 5 seconds at 10-minute intervals per chamber. Locomotor activity was recorded every second in the X- and Z-axes. Airflow through the chambers was regulated to maintain an oxygen differential of approximately 0.3% under resting conditions. The respiratory exchange ratio (RER) was calculated as VCO_2/VO_2 . Total energy expenditure, carbohydrate oxidation, and lipid oxidation were derived from VO_2 , VCO_2 , and estimated protein catabolism using established formulas.

RNA-sequencing and data analysis

RNA was isolated from liver samples as described above. Samples were quantitated with a Qubit RNA assay (ThermoFisher Scientific) and RNA quality was determined with the Agilent TapeStation RNA assay (Agilent Technologies). Libraries were prepared with the Stranded mRNA Prep, Ligation Kit (Illumina). One μg of RNA was processed for each sample and mRNA was purified and fragmented. cDNA was synthesized, and 3' ends were adenylated. Anchor sequences were ligated to each sample, and a limited-cycle PCR was performed to amplify and index the libraries. The average library size was determined using an Agilent TapeStation D1000 assay (Agilent Technologies) and libraries were quantitated with qPCR (Bio-rad CFX96 Touch Real-Time PCR, NEB Library Quant Kit for Illumina). Libraries were normalized to 0.5 nM and pooled. The library pool was denatured and diluted to approximately 100 pM. A 1% library of 2.5pM PhiX was spiked in as an internal control. Paired end 76 x 76 base pair sequencing was performed on an Illumina NovaSeq 6000. Primary analysis, including base calling and quality scoring, was performed onboard the Illumina NovaSeq 6000 (NovaSeq Control Software v1.8.0; RTA v3). Samples were de-multiplexed, the adapter sequences were removed (the first 9 cycles of sequencing were trimmed), and FASTQ files were generated. RNA-seq FASTQ reads were aligned to the mouse reference genome and transcriptome (GRCm38) using STAR (v2.7.10a) (19) and quantified with RSEM (v1.3.1), following the nf-core/rnaseq pipeline (v3.14.0) (20). Differential expression analysis was carried out in R (v4.4.1) using the limma + voom (v3.60.6) and edgeR (v4.2.2) packages (20, 21). Multiple testing correction was applied using the Benjamini-Hochberg procedure, and genes were considered significantly differentially expressed at $p < 0.05$ (raw) and $|\log_2(\text{fold change})| \geq 0.5$. Hierarchical clustering of differentially expressed genes was performed and visualized as heatmaps using the ComplexHeatmap package (v2.20.0). Gene set enrichment analysis (GSEA) was conducted with clusterProfiler (v4.12.6) (22), referencing the Gene Ontology (GO), KEGG, and Reactome databases (23). Adjusted p -values were again calculated using the Benjamini-Hochberg method.

In silico docking analysis

The target protein PPAR α (PDB ID: 4BCR) was extracted from the Protein Data Bank (PDB). The structure of ligands 8(S)-hydroxyeicosatetraenoic acid (8S-Hete), Leukotriene B₄, and C18:1-Leu were obtained from PubChem. Initially, the PDB files of selected protein were processed by deleting ligands and non-proteins using Discover Studio visualizer v20.1.0.19295. Further, the protein structure was prepared by removing water molecules and then the addition of Kollman charges and polar hydrogen atoms. Next, the ligands were processed by merging Gastiger charges to their structures. Finally, both ligand and protein structures were converted to pdbqt file using AutoDock Tools 1.5.6. AutodockVina v1.1.2 carried out docking calculations. The dimension of the grid used for both the proteins was kept 40X40X40 Å³ and separated by 0.375 Å grid-point spacing. The exhaustiveness for the docking run was set to the default value of eight. The ligand's binding energy was calculated using the default Lamarckian genetic algorithm of the AutoDock Vina software package. After completing the docking run, the protein-ligand complexes were analyzed using Biovia Discovery Studio Visualizer v20.1.0.19295 and Pymol molecular graphics software. Biovia Discovery Studio Visualizer v20.1.0.19295 generated the representative 2D and 3D structures showing protein-ligand structures.

Transwell chemotaxis assay

HepG2 cells were plated at 1×10^5 cells per well of a 24 well plate and transfected with 20 nM of siRNA against CCL2 (siCCL2, CCL2 Human ON-TARGETplus siRNA, Dharmacon, #L-007831-00-000) or scrambled siRNA (siCTL, ON-TARGETplus non-targeting pool, Dharmacon, CO #D-001810-10-05) using Lipofectamine RNAiMAX (Invitrogen #13778150) in Opti-MEM reduced-serum medium (Gibco) in

accordance with the manufacturer's protocol. After 24 hours, cells were treated with C18:1 or C18:1-Leu (10 μ M) in the presence of BSA-PA (200 μ M). hPBMs were labeled with Vybrant DiO cell-labeling solution and suspended at approximately 5×10^6 cells/mL of warmed HBSS. Millicell cell culture hanging inserts with 8 μ m pores (Millipore #PTEP24H48) were inserted into each well, and 100 μ L of hPBMs were added to the top well of each insert. hPBMs were permitted to incubate for approximately 18 hours in the insert and pass through the pores into the bottom well. Following hPBM transmigration, inserts were carefully removed and the bottom well containing both HepG2 cells and hPBMs were fixed in 3.7% neutral buffered formalin for at least 20 min. Cells were visualized on a Keyence BZ-X810 all-in-one fluorescence microscope, and the total number of hPBMs that passed into the bottom well were quantified using Keyence BZ-X800 analyzer software. Representative images of the fluorescent hPBMs (shown in green) were taken with a brightfield overlay to visualize equal numbers of HepG2 cells for each treatment.

Chromatin immunoprecipitation (ChIP) assay

To measure the binding between p65 protein and binding sites in the promoter DNA of the mouse *Ccl2* gene (−2374: GGGAATTTCC; −2348: GGGAATTCC), primary hepatocytes were isolated from 8-10-week-old *Ppara*^{fl/fl} and *Ppara*^{HKO} male mice maintained on a SD. The primary hepatocytes were treated with C18:1 or C18:1-Leu (10 μ M) in the presence of BSA-PA (200 μ M). We utilized ChIP antibody against NF- κ B p65 (Sigma-Aldrich, #CS204359) and rabbit immunoglobulin G (IgG) as control (Sigma-Aldrich: #CS200621). The ChIP procedure was performed as per the manufacturer's protocol (Cell Signaling Technology, 9003S). The qPCR was performed for detecting the co-immunoprecipitated DNA containing the functional p65-binding sites. The ChIP primers utilized for qPCR are as follows: forward: 5'-TGCTTGGCTGCAGGCCAG-3', reverse: 5'-AGGATGTTCTTCCCAGCGG-3' (24).

Statistics

Statistical analyses were performed using GraphPad Prism 10.6.0. All data were tested for normality. If passed, Student's t test was used to compare two groups or one-way analysis of variance (ANOVA) followed by Tukey post hoc test for comparisons among >2 groups. Otherwise, nonparametric tests (Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's post hoc test) were used. Spearman's correlations between hepatic NAA levels, NAS and fibrosis scores were evaluated. A p value <0.05 was considered statistically significant.

Study approval

All animal procedures were approved by the Institutional Animal Care and Use Committees of Louisiana State University Health Shreveport (P-21-043, P-22-035, and P-24-024) and the University of Michigan (PRO00008239). All animal studies were conducted in accordance with the institutional guidelines and applicable regulations.

For the human studies, deidentified liver specimens from the early MASLD and end-stage MASH cohorts were collected as previously described (10, 11 and 4, respectively). Collection and use of human specimens were approved by the Institutional Review Board of the Ochsner Clinic Foundation (protocols 2010.179, 2016.131.B, and 2020.039). Human studies were conducted through the Ochsner Multi-Organ Transplant Institute with specimens collected following informed consent.

Supplemental references

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