

# A host enzyme reduces non-alcoholic fatty liver disease by inactivating intestinal lipopolysaccharide

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## Abstract

The incidence of Non-alcoholic Fatty Liver Disease (NAFLD) has been increasing world-wide. Since gut-derived bacterial lipopolysaccharides (LPS) can travel via the portal vein to the liver and play an important role in producing hepatic pathology, it seemed possible that (1) LPS stimulates hepatic cells to accumulate lipid, and (2) inactivating LPS can be preventive. Acyloxyacyl hydrolase (AOAH), the eukaryotic lipase that inactivates LPS and oxidized phospholipids, is produced in the intestine, liver, and other organs. We fed mice either normal chow or a high-fat diet for 28 weeks and found that *Aoah*<sup>-/-</sup> mice accumulated more hepatic lipid than did *Aoah*<sup>+/+</sup> mice. In young mice, before increased hepatic fat accumulation was observed, *Aoah*<sup>-/-</sup> mouse livers increased their abundance of Sterol Regulatory Element-Binding Protein 1 (SREBP1) and the expression of its target genes that promote fatty acid synthesis. *Aoah*<sup>-/-</sup> mice also increased hepatic expression of CD36 and Fabp3, which mediate fatty acid uptake, and decreased expression of fatty acid-oxidation-related genes *Acot2* and *Ppar-α*. Our results provide evidence that increasing AOAH abundance in the gut, bloodstream and/or liver may be an effective strategy for preventing or treating NAFLD.

## eLife Assessment

This **important** study highlights the key role of the gut-liver axis mediated by LPS in causing hepatic steatosis. The authors provide **solid** evidence, in vivo, in vitro, and in silico, for the role of acyloxyacyl hydrolase in mediating this effect using KO mice subjected to MASD-inducing diets. The findings are significant for the liver research community and others interested in the gut-liver axis.

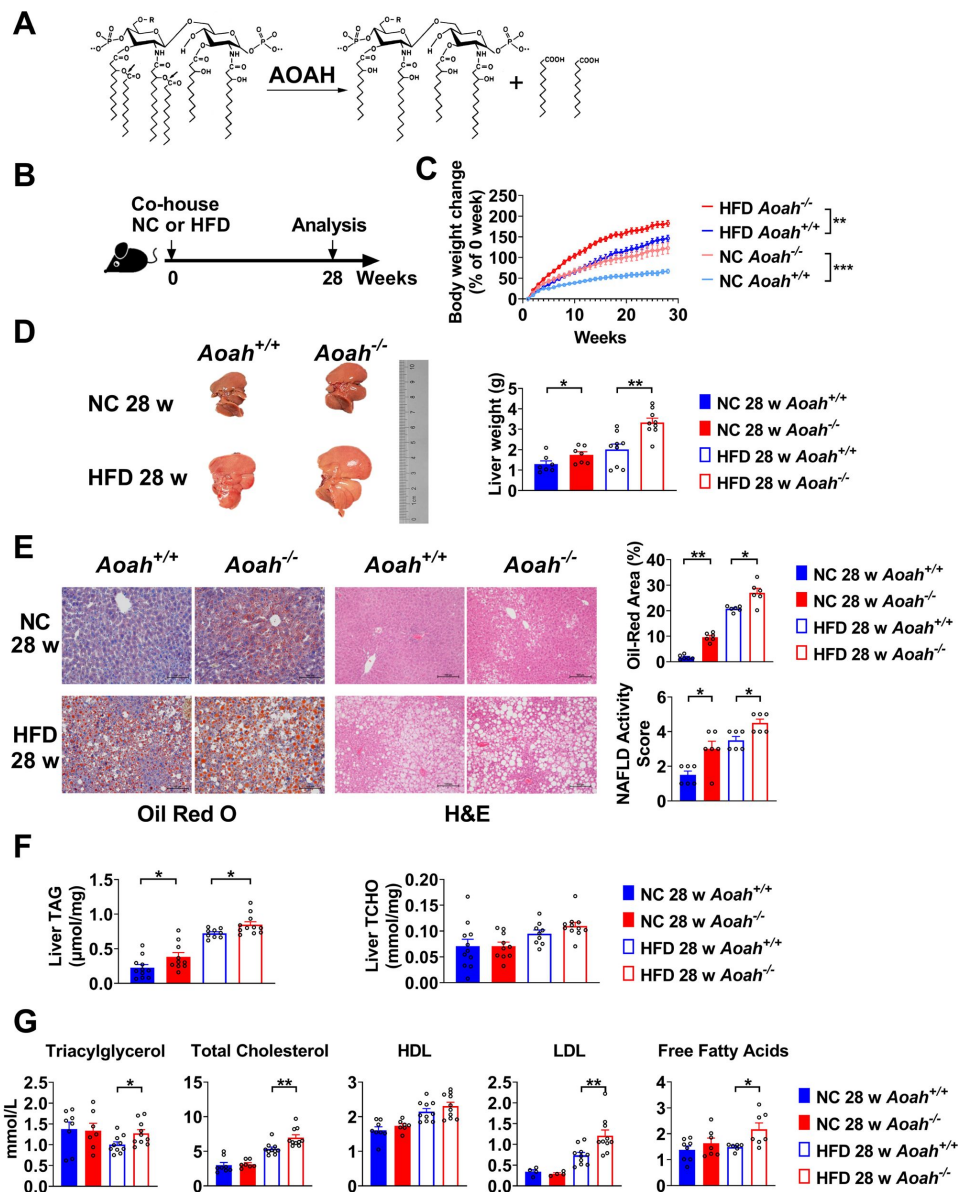
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## Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is a common human affliction. Its global prevalence, currently about 25%, has been increasing (Diehl and Day, 2017; Fan et al., 2017; Younossi et al., 2018). NAFLD may progress from nonalcoholic fatty liver to nonalcoholic steatohepatitis, cirrhosis and even hepatic cancer (Friedman et al., 2018; Sheka et al., 2020). Multiple factors may contribute to its pathogenesis (Friedman et al., 2018); prominent among these are the lipopolysaccharides (LPSs, endotoxins) produced by many of the Gram-negative bacteria that inhabit the intestine. Gut-derived LPS may translocate into the portal venous system and traffic to the liver, triggering or exacerbating hepatic inflammation (Albillos et al., 2020; Carpino et al., 2020; Han et al., 2021; Kazankov et al., 2019; Leung et al., 2016; Munford, 1978; Wang et al., 2022).

The LPS molecules that contribute to NAFLD pathogenesis are able to stimulate host cells because their lipid A structure is recognized by MD-2/TLR4 receptors (Ye et al., 2012). Most *γ-Proteobacteria* such as *E. coli* produce stimulatory hexaacyl LPS (with six acyl chains) while *Bacteroidetes* produce non-stimulatory LPS that has four or five acyl chains (Anhe et al., 2021; d’Hennezel et al., 2017). NAFLD is often associated with intestinal dysbiosis produced by increased abundance of *γ-Proteobacteria*, which leads to tissue inflammation and increased intestinal permeability that allows even more gut-derived LPS to reach the liver (Albillos et al., 2020; Aron-Wisnewsky et al., 2020a; Aron-Wisnewsky et al., 2020b; Mouries et al., 2019; Rodrigues et al., 2024). Although gut-derived LPS is known to induce hepatic inflammation and to exacerbate NAFLD, how LPS influences hepatocyte fatty acid metabolism before NAFLD develops has not been well understood.

Acyloxyacyl hydrolase (AOAH) is a highly conserved animal lipase that is mainly expressed in macrophages, monocytes, neutrophils, microglia, dendritic cells, NK cells and ILC1 cells (Munford et al., 2020). It can inactivate Gram-negative bacterial LPSs by releasing two of the six fatty acyl chains present in the lipid A moiety (Fig 1A). It also can deacylate/inactivate oxidized phospholipids and lysophospholipids, molecules that are also known to contribute to NAFLD (Sun et al., 2020; Zou et al., 2021). We previously reported that AOAH is expressed by gut macrophages and dendritic cells and can inactivate bioactive LPS in feces (Cheng et al., 2023; Janelins et al., 2014; Qian et al., 2018). Han et al. found that intestine-derived LPS can bind high-density lipoprotein 3 (HDL<sub>3</sub>) and be inactivated by AOAH as it traffics to the liver via the portal vein (Han et al., 2021). AOAH also inactivates LPS in the liver, diminishing and shortening hepatic inflammation induced by bloodborne (i.v. injected) LPS (Ojogun et al., 2009; Shao et al., 2011; Shao et al., 2007). The enzyme’s ability to prevent NAFLD by inactivating gut-derived LPS had not been tested (Ojogun, 2008).



**Fig 1.**

### AOAH reduces hepatic lipid accumulation.

(A) AOA cleaves the two piggyback fatty acyl chains from the lipid A moiety, inactivating LPS. The arrows indicate the cleavage sites.

(B) Co-housed *Aoah*<sup>+/-</sup> and *Aoah*<sup>-/-</sup> mice were fed either a normal diet (NC) or a high fat diet plus high fructose (23.1 g/L) and glucose (18.9 g/L) in their drinking water (HFD) for 28 weeks.

(C) Body weight was measured weekly for 28 weeks. Data were combined from 4 experiments. n = 12 – 17.

(D) Representative images of livers at 28 weeks are shown and the liver weight was measured. n = 7 – 9.

(E) Mouse livers were fixed, sectioned and stained with Oil Red O or Hematoxylin-eosin (H & E). In Oil Red O-stained sections, the percentage of area occupied by the lipid droplets was quantified using Image J. H & E staining results were semi-quantitatively scored for disease severity. Data were combined from 3 experiments, n = 6.

(F) Triacylglycerol (TAG) and total cholesterol (TCHO) were measured in liver homogenates. Data were combined from 3 experiments, n = 9 – 11.

(G) The serum concentrations of triglyceride, total cholesterol, HDL, LDL and free fatty acids were measured. Data were combined from 3 experiments. n = 6 – 10.

(C - G) Mann-Whitney test and Two-way ANOVA test were used. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.

In this study, we found that when mice were fed either normal chow or a high fat diet, AOA reduced LPS-induced lipid accumulation in the liver, probably by decreasing the expression and activation of Sterol Regulatory Element-Binding Protein 1 (SREBP1), an important transcription factor that promotes fatty acid synthesis (Horton et al., 2002 [DOI](#); Shimano and Sato, 2017 [DOI](#)). AOA also reduced the expression of CD36 and *Fabp3*, fatty acid uptake-related genes, and increased that of fatty acid oxidation-related genes (*Acot2* and *Ppar-α*). In addition, AOA reduced hepatic inflammation and minimized tissue damage. Our results suggest that AOA plays a regulatory role in ameliorating NAFLD and suggest that measures that increase AOA abundance in the intestine, liver and/or bloodstream may help prevent this common disease.

## Materials and methods

### Mice

C57BL/6J *Aoah*<sup>-/-</sup> mice were produced at the University of Texas Southwestern Medical Center, Dallas, Texas (Lu et al., 2003 [DOI](#)), transferred to the National Institutes of Health, Bethesda, Maryland, USA, and then provided to Fudan University, Shanghai, China. The mutated *Aoah* gene had been backcrossed to C57BL/6J mice for at least 10 generations. *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> male mice were housed in a specific pathogen-free facility with 12-h light/dark cycle at 22 °C in Fudan University Experimental Animal Center (Shanghai, China). *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> male mice were co-housed for at least 3 weeks before the start and throughout the experiments. All studies used protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Fudan University (2023-DWYY-03JZS). All animal study protocols adhered to the Guide for the Care and Use of Laboratory Animals.

### The NAFLD mouse model

Co-housed *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> male mice were fed either a normal diet (NC) or a high fat calorie diet (D12492, Research Diets, USA) that contained protein: carbohydrate: fat (20:20:60, kcal%) plus high fructose (23.1 g/L; F3510, Sigma, USA) and glucose (18.9 g/L; G8270, Sigma, USA) in the drinking water (HFD) for 28 weeks (Liu et al., 2018 [DOI](#)).

### Blood analysis

Total triacylglycerol, total cholesterol, low density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in mouse serum were measured in the Department of Laboratory Animal Science, Fudan University using ADVIA Chemistry XPT.

### Liver histology

After livers were excised and fixed in 4% paraformaldehyde for 18 h, they were sectioned and stained with hematoxylin and eosin (H & E) and Oil Red O. The samples were examined for steatosis, hepatocyte ballooning degeneration, lobular inflammation and lipid droplets using a Nikon E200 microscope.

### NAFLD activity score

Histological analysis of the liver was performed based on the Sheka NAFLD scoring criteria (Sheka et al., 2020 [DOI](#)). Liver steatosis is an infiltration of hepatic fat with minimal inflammation and is graded based on the fat percentage in hepatocytes: grade 0 (< 5%), grade 1 (5% – 33%), grade 2 (33% – 66%), and grade 3 (> 66%). Inflammatory activity is manifested by two factors: grade 0 (no inflammation), grade 1 (< 2 foci per 200 × field), grade 2 (2 – 4 foci per 200 × field), grade 3 (> 4 foci per 200 × field), and the presence of hepatocyte ballooning degeneration: no ballooned cells (grade 0), a few ballooned cells (grade 1), and many ballooned cells (grade 2).

## Liver lipid analysis

Undiluted serum samples and liver homogenates (50 mg/ml) were run in duplicate alongside a standard curve of glycerol (triglyceride assay), cholesterol (cholesterol assay) or palmitic acid (free fatty acid assay) according to the manufacturer's instructions. Triglyceride Determination Kit and Cholesterol Determination Kit were obtained from Applygen. Free Fatty Acid Quantitation Kit was purchased from Sigma-Aldrich.

## Real time-PCR (qPCR)

RNA from livers or isolated hepatocytes was purified using TRNzol Universal Reagent (Tiangen) and reversely transcribed (Tiangen). The primers used for qPCR are listed in Table S1. Actin was used as an internal control and the relative gene expression was calculated using the  $\Delta\Delta C_t$  quantification method.

## Liver immune cell isolation

After mice were exsanguinated, 2 ml PBS containing collagenase (5 mg/ml, type IV, Sigma) were injected into the liver via the inferior vena cava. The liver was cut into small pieces and treated with collagenase (0.5 mg/ml) for 10 min at 37 °C. The liver pieces were mashed by using syringe plungers. The cells were passed through a 70  $\mu$ m strainer (WHB scientific) and then centrifuged at 50 g for 3 min, 3 times, to pellet hepatocytes. The immune cells in the supernatant were pelleted (500 g for 15 min) and then isolated on a 40% Percoll step gradient (Cytiva). The cells were then stained and analyzed using flow cytometry (Shao et al., 2007 [\[4\]](#)).

## Flow cytometry

Liver cells were collected by centrifugation and then incubated with Fc blocking antibody (purified anti-mouse CD16/32, BioLegend) on ice for 15 min. After the cells were stained with fluorescence-conjugated antibodies for 30 min on ice, they were washed and subjected to FACS (BD, FACSCelesta). The FACS data were analyzed using Flow Jo software (TreeStar, Inc). All antibodies used for flow cytometry were anti-mouse antigens. Anti-mouse antibodies used for flow cytometry were anti-CD45-BV785 (Clone 30-F11, BioLegend), anti-CD11b-FITC (Clone M1/70, BioLegend), anti-F4/80-BV421 (Clone BM8, BioLegend), anti-Ly6G-PerCP-Cy5.5 (Clone 1A8, BioLegend), anti-Ly6C-APC-Cy7 (Clone HK1.4, BioLegend), anti-MHC II-PE-Cy7 (Clone M5/114.15.2, BioLegend), anti-VSIG4-APC (Clone NLA14, eBioscience), and anti-Tim4-PE (Clone RMT4-54, BioLegend).

## Hepatocyte isolation

Primary hepatocytes were isolated from adult mice using a two-step collagenase perfusion method (Charni-Natan and Goldstein, 2020 [\[4\]](#)). In brief, the peritoneal cavity was opened and the liver was perfused *in situ* via the portal vein at 37°C with 20 ml PBS followed by 20 ml DMEM containing 10 mg collagenase (type IV, Sigma). The liver was then removed and gently minced, and the released cells were dispersed in DMEM containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The solution containing the mixed cells and debris was passed through a 100  $\mu$ m cell strainer, and then centrifuged at 50 g for 3 min, twice. The hepatocytes were pelleted and then isolated on a 90% Percoll step gradient (Cytiva). Hepatocytes were then resuspended in a TRNzol Universal Reagent (Tiangen) to measure mRNA.

## Western blot

Small pieces of liver were lysed with RIPA buffer (Biyotime) containing 1 mM PMSF (Biyotime) and a proteinase inhibitor mixture (Selleck). A commercial cytosol and nucleus Protein Extraction Kit (P0027, Beyotime) was used to separate cytosolic and nuclear proteins in the liver. The following antibodies were used for Western analysis: anti-SREBP1 (SC-17755; Santa cruz), anti-AOAH

(HPA021666; Sigma-Aldrich), anti-Lamin B1 (12987-1-AP, Proteintech), anti-Phospho-mTOR (Ser2448) (5536, Cell Signaling Technology), anti-Phospho-p70 S6 Ribosomal Protein (Thr389) (9234, Cell Signaling Technology), anti-Phospho-AKT (Ser473) (4060, Cell Signaling Technology), anti-Phospho-mTOR (Ser2448) (5536, Cell Signaling Technology), anti-mTOR (2983, Cell Signaling Technology), anti-p70 S6 Ribosomal Protein (2217, Cell Signaling Technology), anti-AKT (4691, Cell Signaling Technology), and anti- $\alpha$ -Tubulin (HRP-66031; Proteintech). Anti-mouse IgG (7076S; Cell Signaling Technology) and anti-Rabbit IgG (7074S, Cell Signaling Technology) were used as secondary antibodies. ECL substrate (Bio-Rad Diagnostic) was used to detect proteins in Western blotting and the blot bands were quantified by using Image J.

## Measurement of TLR4-stimulating activities in mouse feces, liver and plasma

Fresh feces were collected and resuspended in endotoxin-free PBS (0.1 g/ml) and centrifuged at 800 g for 5 min. The supernatant was heated at 70 °C for 10 min. Mice were bled from the eye socket; 5  $\mu$ l of 0.5 M EDTA was used as an anti-coagulant. Livers were homogenized in PBS, centrifuged and the supernatant was obtained. In some experiments, 200  $\mu$ g of LPS was resuspended in 200  $\mu$ l of PBS and then the suspension was slowly administered into the esophagus of mice using a gavage needle. Twenty-four h later, the livers were collected for analysis. All the samples were collected for TLR4-stimulating activity using a cell-based colorimetric endotoxin detection kit (HEK-Blue LPS Detection Kit2, InvivoGen). Diluted samples were added to human embryonic kidney (HEK-293) cells that expressed hTLR4 and a NF- $\kappa$ B-inducible secreted embryonic alkaline phosphatase reporter gene. After 18 h incubation, cell culture media were applied to QUANTI-Blue medium to measure alkaline phosphatase activity. A preparation of *E. coli* 055:B5 LPS, standardized to FDA-approved control standard endotoxin, which was included in the kit, was used to quantitate TLR4-stimulating activity. Plates were read at a wavelength of 620 nm (Tecan).

## Gut permeability analysis

After mice were fasted for 18 h, they were orally gavaged with fluorescein isothiocyanate (FITC)-conjugated 4 kDa dextran (50 mg per 100 g body weight) (46944, Sigma-Aldrich). Four h after gavage, blood was collected from the facial vein and the serum was used for FITC fluorescence measurements (excitation, 490 nm; emission, 520 nm).

## RNA-sequencing analysis

Total RNA was isolated using TRNzol from co-housed 6 - 8 weeks old *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mouse livers. The libraries were sequenced on an Illumina Novaseq 6000 platform and 150 bp paired-end reads were generated. Differential expression analysis was performed using the DESeq2. Q value < 0.05 and foldchange > 1.5 was set as the threshold for significantly differential gene expression. Gene Set Enrichment Analysis (GSEA) was performed using GSEA software. RNA-seq analysis was conducted by Shanghai OE Biotech. Co., Ltd. China and the results were deposited at PRJNA1022016.

## Statistical analysis

Data are presented as mean  $\pm$  SEM. Differences between groups were analyzed using Mann-Whitney test. To compare kinetic difference, two-way ANOVA test was used. The statistical significance was set at  $P < 0.05$ . \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

## Results

### AOAH reduces hepatic lipid accumulation

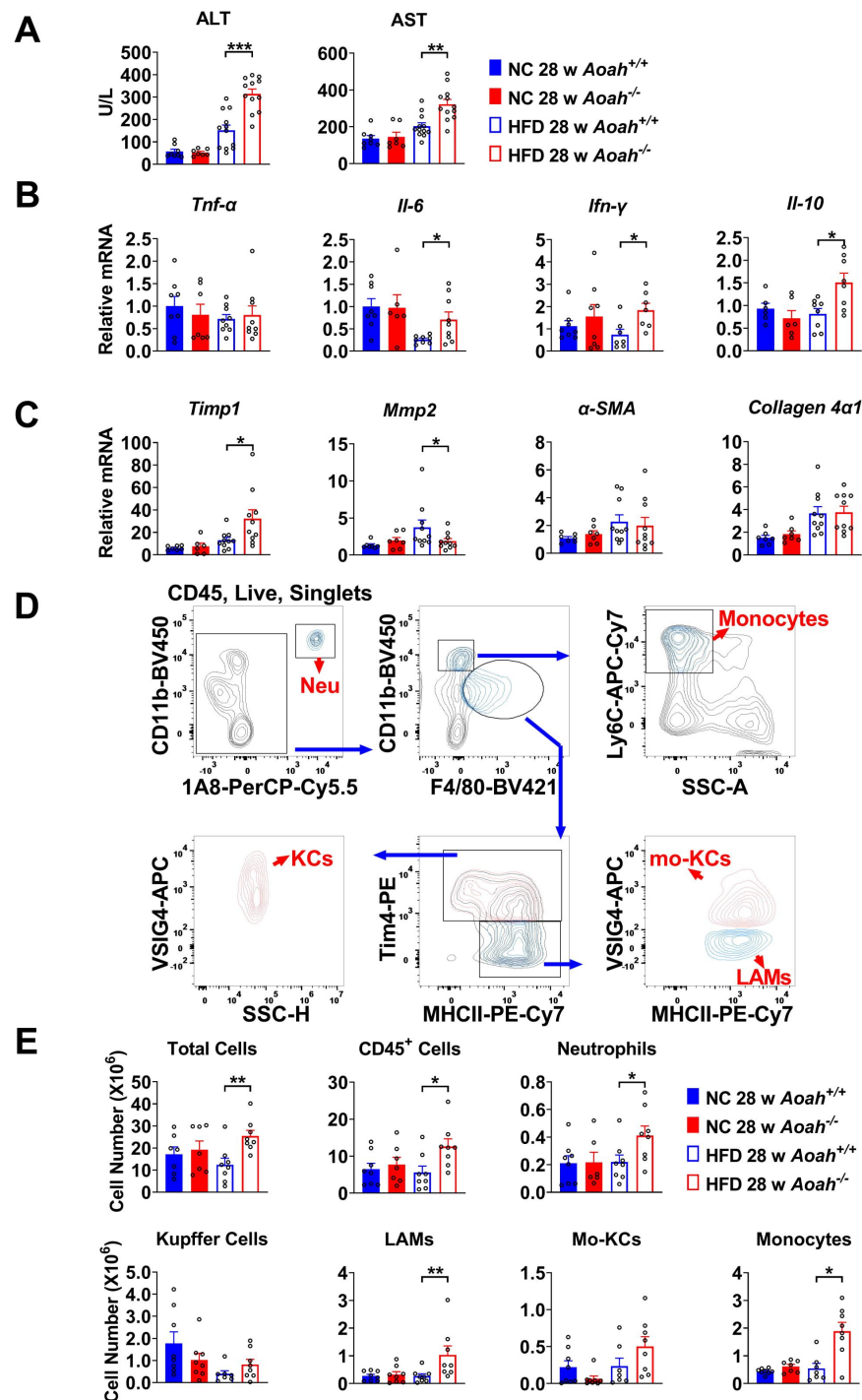
To find out if AOAH prevents NAFLD, we fed co-housed *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice Normal Chow (NC) or a High Fat Diet (HFD), supplemented with fructose and glucose in the drinking water, for 28 weeks (**Fig 1B**) (Liu et al., 2018). *Aoah*<sup>-/-</sup> mice fed either NC or HFD gained more weight than did respective *Aoah*<sup>+/+</sup> control mice (**Fig 1C**). When they were fed either NC or HFD, the livers of *Aoah*<sup>-/-</sup> mice were heavier than those of *Aoah*<sup>+/+</sup> control mice (**Fig 1D**). Histological examination and Oil Red O staining revealed that when they were fed NC or HFD, *Aoah*<sup>-/-</sup> mouse livers accumulated more lipid droplets than did the livers of *Aoah*<sup>+/+</sup> mice (**Fig 1E**). When we scored NAFLD intensity based on steatosis, hepatocyte ballooning degeneration and inflammation, *Aoah*<sup>-/-</sup> mice developed more severe NAFLD than did *Aoah*<sup>+/+</sup> mice whether the mice were fed NC or HFD (Sheka et al., 2020) (**Fig 1E**). When the mice were fed either NC or HFD, *Aoah*<sup>-/-</sup> mouse livers contained more triacylglycerol (TAG) than did *Aoah*<sup>+/+</sup> mouse livers, while livers from both mouse strains contained a similar amount of total cholesterol (TCHO, **Fig 1F**). When the mice were fed the HFD, *Aoah*<sup>-/-</sup> mice had higher serum levels of triacylglycerol, total cholesterol, low density lipoprotein (LDL) and free fatty acids than did *Aoah*<sup>+/+</sup> mice (**Fig 1G**). Collectively, these findings were evidence that AOAH reduced hepatic triacylglycerol accumulation when mice were fed either NC or HFD.

### AOAH prevents hepatic inflammation and tissue injury when mice are fed HFD

*Aoah*<sup>-/-</sup> mice fed HFD had significantly higher serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels than did control *Aoah*<sup>+/+</sup> mice, suggesting that *Aoah*<sup>-/-</sup> mice experienced more severe liver inflammation and tissue damage (**Fig 2A**). To assess liver inflammation, we measured pro- and anti-inflammatory cytokine expression. When mice were fed the HFD, *Aoah*<sup>-/-</sup> mouse livers produced more pro-inflammatory IL-6, IFN- $\gamma$  and anti-inflammatory IL-10 mRNA than did *Aoah*<sup>+/+</sup> mouse livers, suggesting greater inflammation (**Fig 2B**). We also measured the expression of genes that are related to fibrosis and found that *Aoah*<sup>-/-</sup> livers had more Timp1 (a pro-fibrosis gene) mRNA and less MMP2 (an anti-fibrosis gene) mRNA than did *Aoah*<sup>+/+</sup> mouse livers (Kisseleva and Brenner, 2021), indicating that *Aoah*<sup>-/-</sup> mouse livers may be developing more severe fibrosis, although we did not detect fibrosis with Masson staining (**Fig 2C**). We analyzed the myeloid cells in the liver (Daemen et al., 2021) and found that when the mice were fed the HFD, *Aoah*<sup>-/-</sup> mouse livers contained more neutrophils, monocytes and lipid-associated macrophages (hepatic LAMs) (Remmerie et al., 2020; Su, 2002) than did *Aoah*<sup>+/+</sup> mouse livers (**Fig 2D, E**). Collectively, when the mice were fed HFD, the livers of *Aoah*<sup>-/-</sup> mice developed significantly greater inflammatory responses and tissue damage.

### AOAH reduces hepatic LPS levels and the expression of fatty acid synthesis genes

We found previously that intestinal macrophages and DCs express AOAH (Janelins et al., 2014; Qian et al., 2018). To confirm that hepatic cells express AOAH, we first consulted the single cell RNAseq analysis reported by Remmerie et al. (Remmerie et al., 2020), who found that AOAH is expressed in Kupffer cells, monocytes, monocyte-derived cells, NK (circulating NK) and ILC1 (tissue resident NK) cells (Remmerie et al., 2020) (**Fig 3A**). We used flow cytometry to sort Kupffer cells (CD45<sup>+</sup>NK1.1<sup>-</sup> F4/80<sup>hi</sup>CD11b<sup>mid</sup>), monocytes (CD45<sup>+</sup>NK1.1<sup>-</sup> F4/80<sup>mid</sup>CD11b<sup>hi</sup>), NK cells (CD45<sup>+</sup>SSC<sup>lo</sup> NK1.1<sup>+</sup>) (circulating NK and resident ILC1) and we purified the hepatocytes. Using qPCR analysis, we found that AOAH mRNA was present in Kupffer cells, monocytes and NK cells



**Fig 2.**

### AOAH prevents hepatic inflammation and tissue injury when mice are fed HFD.

(A) Serum ALT and AST were measured. Data were combined from 3 experiments, n = 7 – 12.

(B-C) Inflammation-related *Tnf-α*, *Il-6*, *Ifn-γ*, *Il-10* mRNA and fibrosis-related *Timp1*, *Mmp2*,  $\alpha$ -SMA, *Collagen 4a1* mRNA were measured in livers at 28 weeks. Data were combined from 3 experiments, n = 6 – 10.

(D) Gating strategy to identify hepatic neutrophils, monocytes, Kupffer cells, lipid-associated macrophages (LAM) and monocyte-derived Kupffer cells (Mo-KC) subsets. (E) The myeloid cell numbers in *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> livers were calculated using FACS analysis. Data were combined from 3 experiments, n = 6 – 8. Mann-Whitney test was used. \*, P < 0.05; \*\*, P < 0.01.

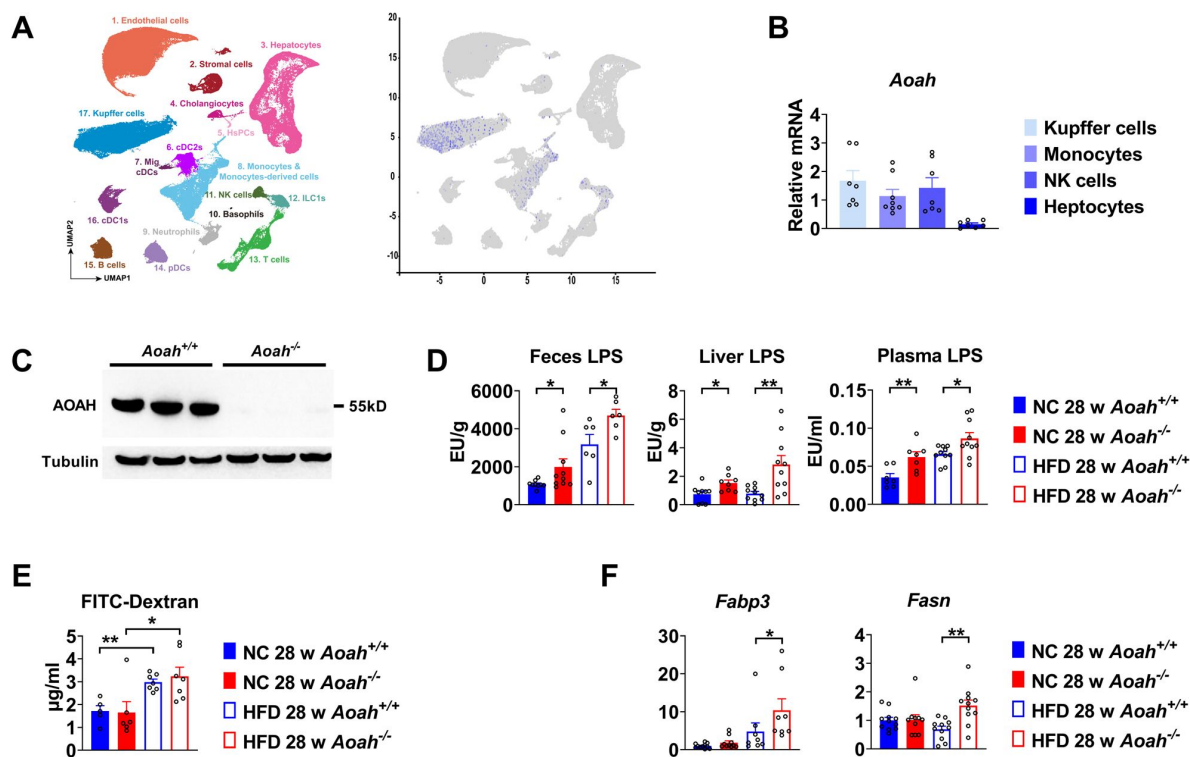
but not in hepatocytes, in keeping with previous findings (Shao et al., 2007) (Fig 3B). Western analysis confirmed that *Aoah*<sup>+/+</sup> mouse livers but not *Aoah*<sup>-/-</sup> mouse livers had AOA protein (Fig 3C). As gut-derived LPS can be transported via the portal vein into the liver (Han et al., 2021), we hypothesized that AOA prevents hepatic inflammation and fat accumulation by inactivating LPS in the gut, portal vein, and liver. We found that *Aoah*<sup>-/-</sup> mouse feces, liver and plasma had higher bioactive LPS levels when *Aoah*<sup>-/-</sup> and *Aoah*<sup>+/+</sup> mice were fed either NC or HFD (Fig 3D). HFD increased gut permeability, but there was no permeability difference between *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice that were fed either NC or HFD (Fig 3E). After the mice were fed HFD for 28 weeks, *Aoah*<sup>-/-</sup> mouse livers increased fatty acid uptake gene *Fabp3* mRNA and fatty acid synthesis gene *Fasn* mRNA, changes that may have contributed to lipid accumulation (Fig 3F).

## AOAH can regulate the expression of hepatic fatty acid metabolism genes

We found that AOA reduced hepatic lipid accumulation when mice were about 8 months old if they were fed either NC or HFD. To investigate the mechanism, we analyzed the livers of young mice. We co-housed 3 - 4 - week - old *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice for 3 - 4 more weeks before removing their livers for RNAseq analysis. The expression of several fatty acid biosynthesis genes, such as *Acacb* (acetyl-Coenzyme A carboxylase beta), *Acsc2* (Acetyl-CoA synthetase 2), *Pcx* (Pyruvate carboxylase), *Acly* (ATP citrate lyase), *Fasn* (Fatty acid synthase) and *Scd1* (Stearoyl-Coenzyme A desaturase 1), was significantly increased in *Aoah*<sup>-/-</sup> mouse livers (Fig 4A). When we used GSEA (Gene set enrichment analysis) to analyze differentially expressed genes, we found that fatty acid biosynthesis pathway was enriched (Fig 4B). We then did qPCR and confirmed increases in mRNAs for FA biosynthesis genes described in Fig 4A as well as for *Acaca* (acetyl-Coenzyme A carboxylase alpha, encoding ACC1, the first and key enzyme on FA synthesis pathway) in *Aoah*<sup>-/-</sup> mouse livers compared with *Aoah*<sup>+/+</sup> mouse livers (Fig 4C). In addition, CD36 (fatty acid uptake (Chen et al., 2022)) mRNA levels increased in *Aoah*<sup>-/-</sup> mouse livers (Fig 4D) while mRNAs for enzymes involved in fatty acid oxidation (*Acot2* (Acyl-CoA thioesterase 2) and *Ppar-α* (peroxisome proliferator-activated receptor α)) (Bougarne et al., 2018; Moffat et al., 2014) decreased (Fig 4E). We found previously that LPS and other TLR agonists increase lipid accumulation in cultured macrophages by increasing expression of *Acs1* (Acyl-CoA synthetase long-chain family member 1) and *Dgat2* (Diacylglycerol O-acyltransferase 2) and by reducing the production of *Atgl* (Adipose triglyceride lipase, *Pnpla2*) (Huang et al., 2014), yet the livers of *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice had similar levels of *Acs1*, *Dgat2* and *Atgl* (*Pnpla2*) mRNA, suggesting that AOA does not regulate hepatic triacylglycerol metabolism (Fig S1). These results suggest that AOA reduces liver fat accumulation by diminishing the expression of fatty acid synthesis and uptake genes and increasing that of fatty acid oxidation genes.

## AOAH reduces hepatic SREBP1

As *Acaca*, *Fasn* and *Scd1* are all target genes for sterol regulatory element-binding protein 1 (SREBP1), a transcription factor for fatty acid biosynthesis, we next analyzed SREBP1 expression in the liver (Horton et al., 2002; Shimano and Sato, 2017). There are two isoforms of SREBP1, SREBP1a and SREBP1c, and the liver predominantly expresses SREBP1c (Horton et al., 2002). The abundance of SREBP1a and SREBP1c mRNA increased in the livers of young *Aoah*<sup>-/-</sup> mice (Fig 5A). SREBP1 is synthesized as a 125 KDa precursor (full length, fSREBP1) in the endoplasmic reticulum, transferred to the Golgi apparatus, and cleaved sequentially by Site-1 protease and Site-2 protease to generate nuclear SREBP1 (nSREBP1, 68 KDa), which enters the nucleus and activates fatty acid biosynthesis gene transcription (Horton et al., 2002; Shimano and Sato, 2017). The livers mainly had the short form (68 KDa) nSREBP1 protein, which was significantly more abundant in *Aoah*<sup>-/-</sup> mouse livers (Fig 5B). We separated liver cytosol and nuclei and found that the short form SREBP1 was mainly present in nuclei and that *Aoah*<sup>-/-</sup> mouse liver nuclei contained significantly more SREBP1 than did *Aoah*<sup>+/+</sup> mouse liver nuclei (Fig 5C).



**Fig 3.**

### AOAH reduces hepatic LPS levels and the expression of fatty acid uptake and synthesis genes.

(A) AOA expression in different kinds of liver cells based on single cell analysis by Remmerie et al. (Remmerie et al., 2020) is shown.

(B) Kupffer cells, monocytes and NK cells were sorted using flow cytometry and hepatocytes were purified from 6 - 8 weeks old mice. AOA mRNA was measured. Data were combined from 3 experiments, n = 7 - 8.

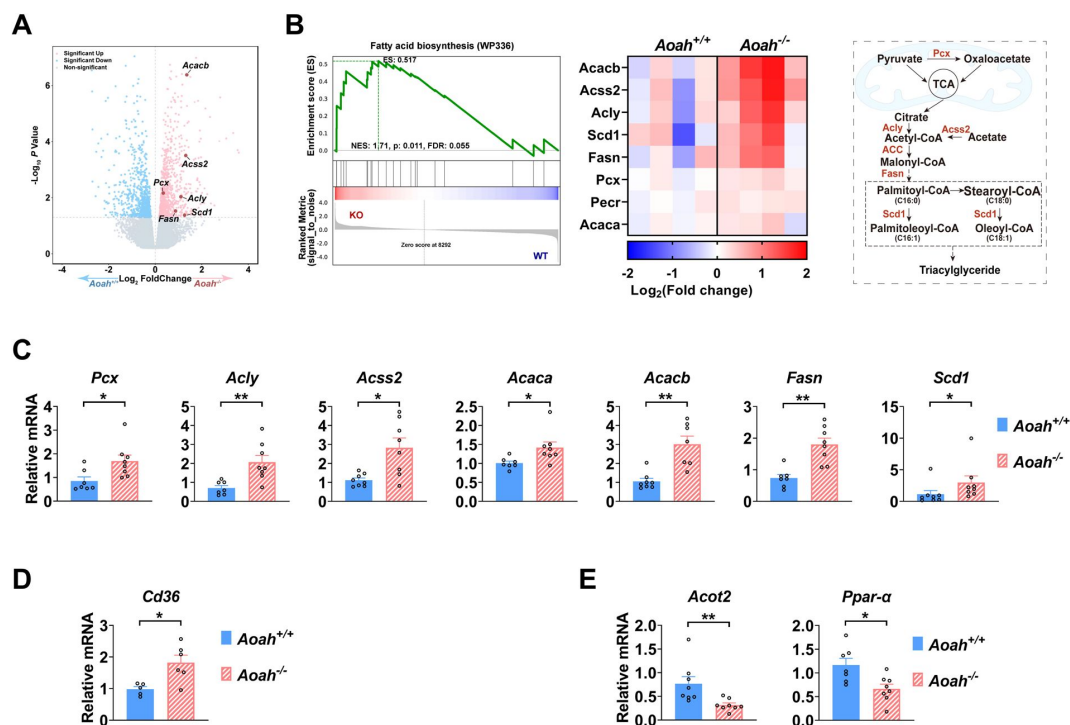
(C) We confirmed that AOA protein was present in 6 - 8-week-old *Aoah*<sup>+/+</sup> mouse livers but not in *Aoah*<sup>-/-</sup> mouse liver homogenates using Western blot. Representative pictures from 3 experiments were shown.

(D) Heated feces suspension, liver homogenates and plasma from *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice were tested for TLR4-stimulating activity. Data were combined from at least 3 experiments, n = 6 - 10.

(E) Gut permeability was measured. Mice were fasted for 18 h. Mice were orally gavaged with fluorescein isothiocyanate (FITC)-conjugated dextran and 4 h later, FITC fluorescence was measured in plasma. Data were combined from 3 experiments, n = 5 - 7.

(F) At 28 weeks of NC or HFD feeding, liver *Fabp3* and *Fasn* mRNAs were measured. Data were combined from 3 experiments, n = 8 - 11.

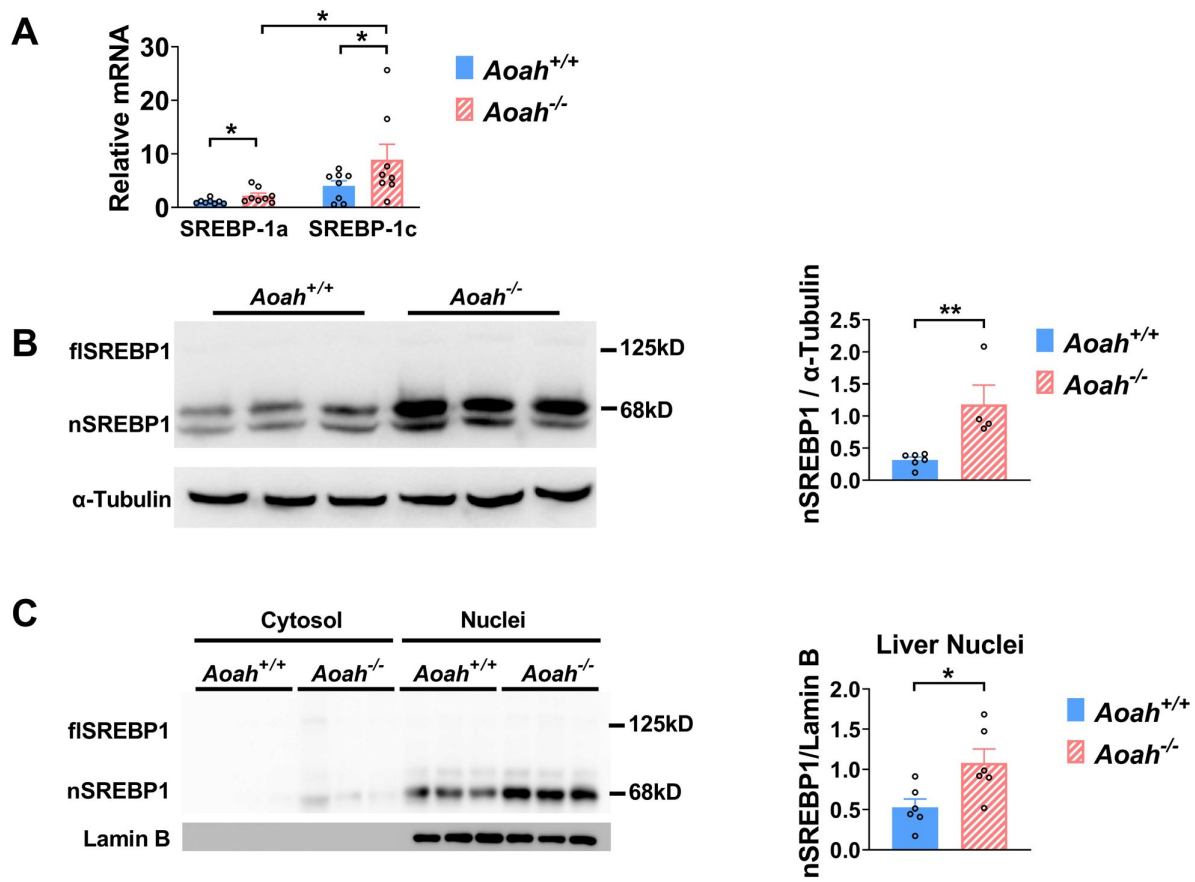
Mann-Whitney test was used. \*, P < 0.05; \*\*, P < 0.01.



**Fig 4.**

### AOAH regulates the expression of hepatic fatty acid metabolism genes.

(A-B) Co-housed 6 – 8 weeks old (i.e. young) *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mouse livers were subjected to RNAseq analysis. The differentially expressed genes were shown in the volcano plot (A). Fatty acid biosynthesis pathway was found enriched using GSEA (Gene set enrichment analysis) (B, left panel). The fatty acid biosynthesis-associated gene expression levels of co-housed *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mouse livers are shown as a heatmap, n = 4 (B, middle panel). The fatty acid biosynthesis pathway is shown. The enzymes marked red had increased expression in young *Aoah*<sup>-/-</sup> mouse livers (B, right panel). (C-E) The hepatic expression of fatty acid synthesis (C), uptake (D) and oxidation (E) genes was measured in co-housed 6 - 8 weeks old *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice. Data were combined from 3 experiments, n = 5 - 8. Mann-Whitney test was used. \*, P < 0.05; \*\*, P < 0.01.



**Fig 5.**

### AOAH reduces hepatic SREBP1.

(A) *SREBP-1a* and *SREBP-1c* gene mRNA was measured in livers from *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice. n = 8.

(B) Liver homogenates from *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice were subjected to Western analysis. SREBP1 protein levels were quantitated using Image J. fSREBP1 is full length SREBP1, which is a precursor; nSREBP1 is nuclear SREBP1, which is an active form. n = 4 – 6.

(C) Liver cytosol and nuclei were separated from *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice and then subjected to Western analysis. SREBP1 protein levels were quantitated using Image J. n = 6.

Mann-Whitney test was used. \*, P < 0.05; \*\*, P < 0.01.

We isolated hepatocytes from 6 - 8 - week - old co-housed *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice and found that *Aoah*<sup>-/-</sup> mouse hepatocytes also expressed higher levels of *Acly*, *Acaca*, *Acacb* and *Fasn* mRNA and lower levels of *Acot2* and *Ppar-α* mRNA, changes that may contribute to lipid accumulation as the mice grow older (Fig S2A - C). When we analyzed the transcript profiles from 6 – 8-week-old co-housed *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mouse livers, we noticed that the expression of serum amyloid A1 (SAA1), SAA2 and SAA3 was significantly higher in *Aoah*<sup>-/-</sup> mouse livers (Fig S2D). Using qPCR, we found increased *Saa1*, *Saa2*, *Saa3* and *Irak-m* mRNA in *Aoah*<sup>-/-</sup> mouse hepatocytes, suggesting that excessive LPS in *Aoah*<sup>-/-</sup> mouse livers induces both inflammatory and regulatory gene expression (Fig S2E). Thus, in young mice, even before hepatic lipid accumulation can be observed, hepatocytes in *Aoah*<sup>-/-</sup> mouse liver have altered expression of genes that may promote lipid storage.

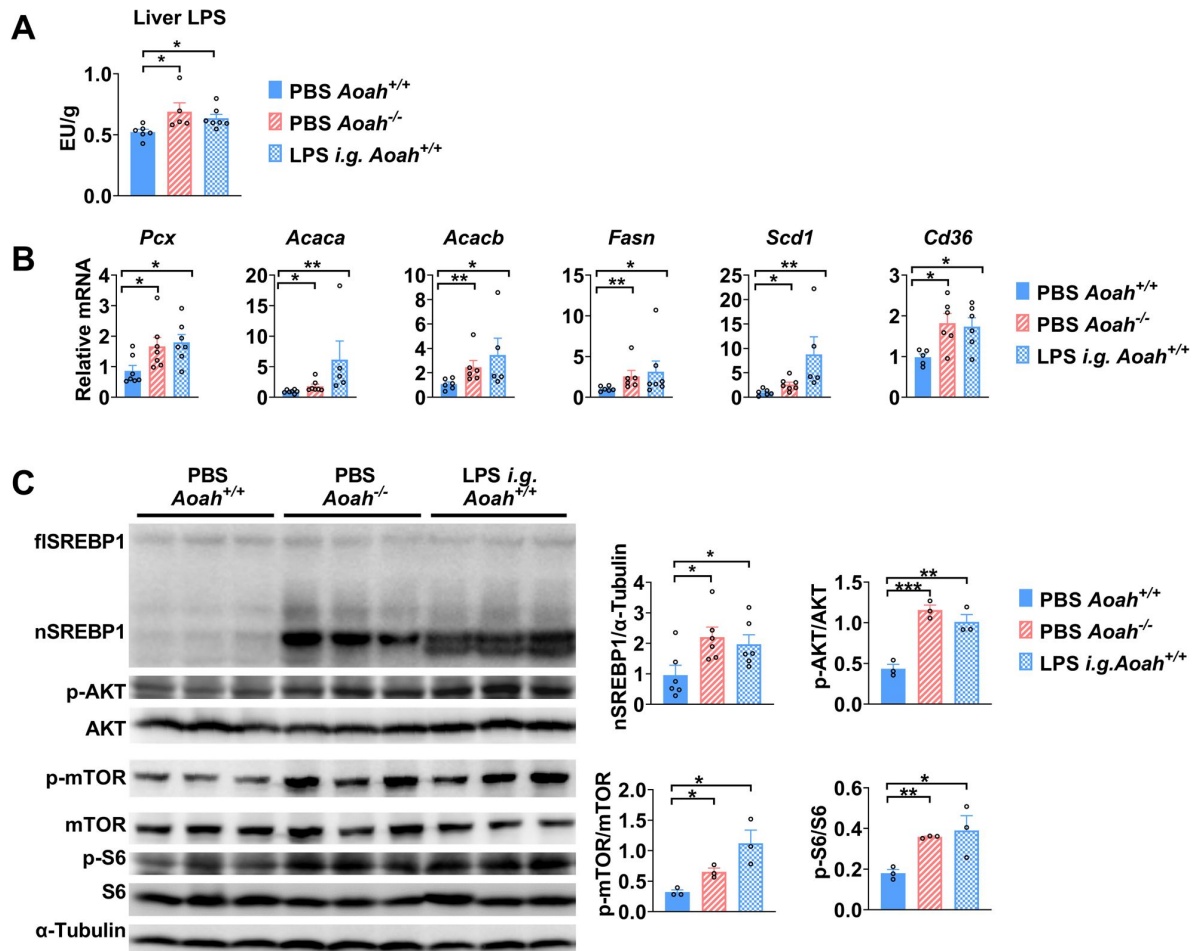
## Excessive gut-derived LPS increases hepatic nSREBP1 and mTOR activation

To test whether excessive gut LPS increases liver LPS levels and promotes fatty acid synthesis gene expression, we orally gavaged *Aoah*<sup>+/+</sup> mice with LPS. We confirmed that orally gavaged (*i.g.*) LPS increased hepatic LPS levels (Fig 6A). Similar to *Aoah*<sup>-/-</sup> mice, *Aoah*<sup>+/+</sup> mice that received gavaged LPS had increased levels of *Pcx*, *Acaca*, *Acacb*, *Fasn*, *Scd1* and *Cd36* mRNA in their livers (Fig 6B). LPS administered *i.g.* also increased nSREBP1 in the liver (Fig 6C). AKT-mTOR1-p70 S6-kinase (S6K) activation induces SREBP1c processing in hepatocytes (Jeon et al., 2023a; Owen et al., 2012; Yecies et al., 2011). Consistently, we found that *Aoah*<sup>-/-</sup> mouse livers had elevated AKT-mTOR-S6K activation (Fig 6C). When we treated *Aoah*<sup>+/+</sup> mice *i.g.* LPS, hepatic AKT-mTOR-S6K activity increased (Fig 6C). These results suggest that excessive hepatic LPS derived from the *Aoah*<sup>-/-</sup> mouse intestine induces mTOR activity, which increases nSREBP1 abundance and fatty acid biosynthesis gene expression in the liver; AOA prevents hepatic lipid accumulation by inactivating gut-derived LPS.

## Discussion

The intestine and liver are connected by the portal vein, enabling the transport of gut commensal-derived molecules, including Gram-negative bacterial LPS, to the liver (Albillos et al., 2020; Leung et al., 2016). Much evidence suggests that gut-derived LPS induces hepatic inflammation and therefore exacerbates NAFLD, especially when dysbiosis and intestinal barrier dysfunction have occurred (An et al., 2022; Aron-Wisnewsky et al., 2020a; Aron-Wisnewsky et al., 2020b; Leung et al., 2016). As lipid accumulation in hepatocytes is considered to be the first hit, gut-derived LPS is usually thought to be the second hit in the pathogenesis of NAFLD, mainly inducing inflammation (An et al., 2022), yet the possibility that LPS also has direct effects on hepatocyte lipid metabolism has received little attention. In previous studies we found that when we co-housed *Aoah*<sup>+/+</sup> and *Aoah*<sup>-/-</sup> mice for 3 or more weeks, they had similar microbiota (Qian et al., 2018), yet we found significantly more LPS in *Aoah*<sup>-/-</sup> mouse feces and livers. *Aoah*<sup>-/-</sup> mice accumulated more hepatic fat than did *Aoah*<sup>+/+</sup> mice when the mice were fed either normal chow or a high fat diet. *Aoah*<sup>-/-</sup> mouse livers also expressed more inflammation-inducing and pro-fibrosis genes and had more liver damage when they were fed a HFD. Notably, when *Aoah*<sup>-/-</sup> mice were young and had not developed NAFLD their livers already expressed significantly elevated levels of nSREBP1 and its target genes (Fig 7).

Liver is an important tissue that converts carbohydrates into lipids (Jeon et al., 2023b). SREBP1c, the predominant isoform expressed in the liver, plays an important role in fatty acid synthesis (Shimano and Sato, 2017); SREBP1c mRNA was elevated in NAFLD patient livers (Kohjima et al., 2008) and its chronic activation contributed to NAFLD progression (Kawano and Cohen, 2013); SREBP1 has become a target for NAFLD treatment (Ju et al., 2020; Jump et al., 2013). Intriguingly, SREBP1 levels increased in the livers of 6 - 8 weeks old (*i.e.*, young) *Aoah*<sup>-/-</sup> mice



**Fig 6.**

### Excessive gut-derived LPS increases hepatic nSREBP1 and mTOR activation.

Livers from *Aoah*<sup>+/+</sup> mice, *Aoah*<sup>-/-</sup> mice and *Aoah*<sup>+/+</sup> mice that were orally gavaged (i.g.) with 200 µg LPS 24 h earlier were obtained.

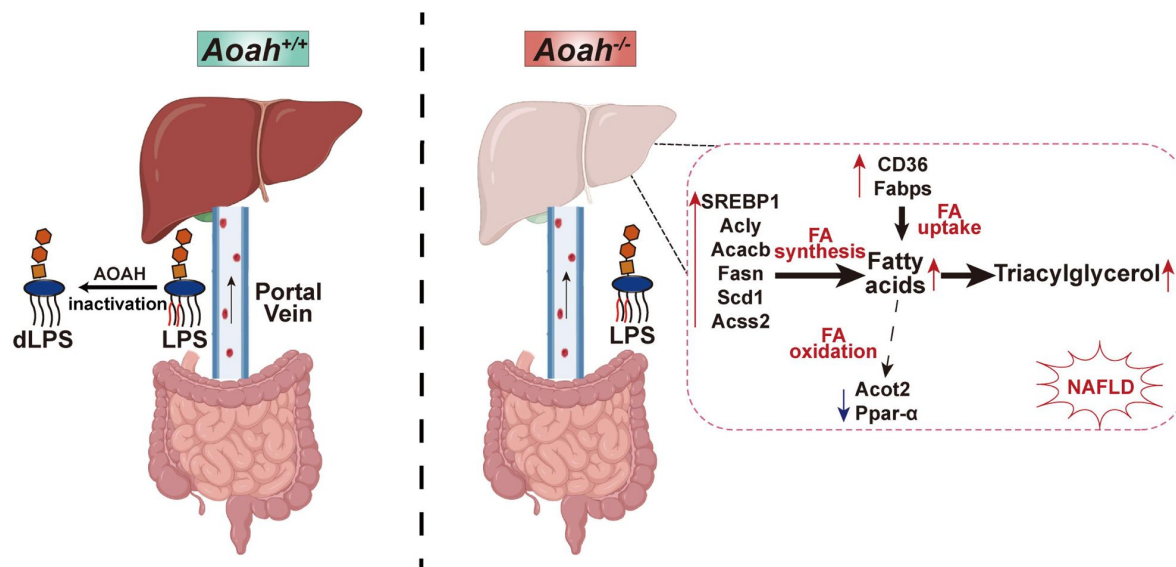
(A) LPS levels were measured.

(B) The mRNA levels of fatty acid biosynthesis genes and CD36 were measured using qPCR.

(C) SREBP1 protein levels and AKT-mTOR activities were measured using Western and Image J. Livers from *Aoah*<sup>-/-</sup> mice and *Aoah*<sup>+/+</sup> mice that were orally gavaged (i.g.) with 200 µg LPS 24 h earlier had increased nSREBP1 and mTOR activities than did those from control *Aoah*<sup>+/+</sup> mice.

Data were combined from 3 experiments, n = 3 – 7.

Mann-Whitney test was used. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.



**Fig 7.**

### AOAH prevents NAFLD by inactivation of gut-derived LPS.

Gut-derived LPS may translocate via the portal vein to the liver. In *Aoah*<sup>+/+</sup> mice, LPS is deacylated in the intestine and portal venous blood; when LPS reaches the liver, LPS is further inactivated by hepatic AOA. In *Aoah*<sup>-/-</sup> mouse liver, gut-derived LPS cannot be deacylated and remains stimulatory. LPS stimulates hepatocytes to generate nuclear SREBP1, which promotes fatty acid biosynthesis gene expression. LPS also increases the expression of fatty acid uptake genes CD36 and Fabps while reducing that of fatty acid oxidation related genes, *Acot2* and *Ppar-α*. As a result of persistent LPS stimulation, *Aoah*<sup>-/-</sup> mice are more susceptible to NAFLD than are *Aoah*<sup>+/+</sup> mice. dLPS = deacylated LPS.

before NAFLD developed; the expression of many SREBP1 target genes, such as those involved in fatty acid biosynthesis (*Acly*, *Acaca*, *Acacb*, *Fasn*, *Scd1*, *Acss2*) also increased (**Fig 7**). We found that orally gavaged LPS increased hepatic LPS, nSREBP1 abundance, and the expression of nSREBP1's target genes, suggesting that gut-derived LPS reaches the liver and promotes fatty acid synthesis. Thus, our data suggest that when AOA is lacking, excessive gut-derived LPS stimulates SREBP1 activation to promote *de novo* lipogenesis in the liver, contributing to more severe NAFLD.

SREBP1a and SREBP1c mRNA both increased in *Aoa*<sup>-/-</sup> mouse livers, suggesting that regulation occurs at the transcription level or because the *Srebp1* gene (encoding SREBP1) promoter contains SREs, increased nSREBP1 induced a feed-forward transcription of SREBP1 (DeBose-Boyd and Ye, 2018). Intriguingly, AKT-mTOR-S6K activity increased in *Aoa*<sup>-/-</sup> mouse livers, which may contribute to increased SREBP1 translocation and processing (DeBose-Boyd and Ye, 2018; Jeon et al., 2023a; Owen et al., 2012). Whether LPS directly stimulates hepatocytes to induce SREBP activation or whether immune cells such as macrophages are involved await further investigation (Barreby et al., 2022; Kazankov et al., 2019; Ye et al., 2012).

In addition to fatty acid biosynthesis gene expression, the expression of CD36, which is involved in free fatty acid uptake (Chen et al., 2022), also increased in the livers of young *Aoa*<sup>-/-</sup> mice while expression of fatty acid oxidation-related genes *Acot 2* and *Ppar-α* decreased (Bougarne et al., 2018; Moffat et al., 2014). In addition to taking up fatty acids, CD36 interacts with INSIG2, a negative regulator of SREBP1, promoting the translocation of SREBP1 from ER to Golgi for cleavage and activation (Zeng et al., 2022). In keeping with our findings, Kim et al., found that LPS suppressed *PPAR-α* expression via ERK activation and HNF4 phosphorylation in primary mouse hepatocyte culture (Kim et al., 2024). In addition to promoting hepatic FA oxidation, *PPAR-α* is a transactivating factor that enhances *Insig2* expression in hepatocytes, preventing SREBP activation (Lee et al., 2017). These results suggest that hepatic LPS stimulation promotes lipid accumulation via many mechanisms. In a previous study, Huang et al., found that LPS and other TLR agonists promoted fat retention in murine macrophages by increasing triacylglycerol synthesis and reducing lipolysis, yet fatty acid synthesis gene abundance did not change (Huang et al., 2014). In contrast, we found that *Aoa*<sup>-/-</sup> and *Aoa*<sup>+/+</sup> mouse livers had similar levels of *Acs1*, *Dgat2* and *Atgl* mRNAs, suggesting that in response to LPS or other PAMPs, hepatocytes and macrophages may accumulate fat via different mechanisms. Notably, we found previously that after LPS stimulation of macrophages *in vitro*, the culture medium became acidic due to aerobic glycolysis (the Warburg effect). The acidic environment contributed more to increasing fat accumulation than did LPS stimulation (Lu et al., 2014). A sensitive pH indicator is needed to find out if the blood and/or extracellular fluid in the liver become acidic due to excessive LPS stimulation and if the acidity promotes hepatic fat accumulation.

In addition to LPS, AOA also deacylates and inactivates oxidized phospholipids and lysophospholipids (Zou et al., 2021), DAMP (Danger-Associated Molecular Pattern) molecules that are induced by inflammation and known to contribute to NAFLD (Sun et al., 2020; Wang et al., 2022). By inactivating gut-derived PAMPs and DAMPs, AOA may decrease hepatic fat accumulation and prevent NAFLD. Increasing AOA abundance may be a useful way to prevent and/or reduce this common disease.

## Data availability

All data are contained within the manuscript and the supplementary files. The RNA-seq data were deposited at PRJNA1022016.

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The authors declare that they have no conflicts of interest with the contents of this article.

## Abbreviations

- AOAH: Acyloxyacyl Hydrolase
- LPS: Lipopolysaccharide
- NAFLD: Non-alcoholic Fatty Liver Disease
- HFD: High Fat Diet
- NC: Normal Chow
- PAMP: Pathogen-Associated Molecular Pattern
- DAMP: Danger-Associated Molecular Pattern
- FA: Fatty Acid
- flSREBP1: full-length Sterol Regulatory Element-Binding Protein 1
- nSREBP1: nuclear SREBP1
- LAM: Lipid-Associated Macrophages
- KCs: Kupffer cells
- Mo-KCs: Monocyte-derived Kupffer Cells.

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## Reviewer #1 (Public review):

Lu *et al.* proposed here a direct role of LPS in inducing hepatic fat accumulation and that the metabolism of LPS therefore can mitigate fatty liver injury. With an Acyloxyacyl hydrolase

whole-body KO mice, they demonstrated that Acyloxyacyl hydrolase deletion resulted in higher hepatic fat accumulation over 8 months of high glucose/high fructose diet. Previous literature has found that hepatocyte TLR4 (which is a main receptor for binding LPS) KO reduced fatty liver in the MAFLD model, and this paper complements this by showing that degradation/metabolism of LPS can also reduce fatty liver. This result proposed a very interesting mechanism and the translational implications of utilizing Acyloxyacyl hydrolase to decrease LPS exposure are intriguing.

The strengths of the present study include that they raised a very simplistic mechanism with LPS that is of interest in many diseases. The phenotype shown in the study is strong. The mechanism proposed by the findings is generally well supported.

There are also several shortcomings in the findings of this study. As AOA<sup>H</sup> is a whole-body KO, the source production of AOA<sup>H</sup> in MAFLD is unclear. Although the authors used published single-cell RNA-seq data and flow-isolated liver cells, physiologically LPS degradation could occur in the blood or the liver. The authors linked LPS to hepatocyte fatty acid oxidation via SREBP1. The mechanism is not explored in great depth. Is this signaling TLR4? In this model, LPS could activate macrophages and mediate the worsening of hepatocyte fatty liver injury via the paracrine effect instead of directly signaling to hepatocytes, thus it is not clear that this is a strictly hepatocyte LPS effect. It would also be very interesting to see if the administration of the AOA<sup>H</sup> enzyme orally could mitigate MAFLD injury. Overall, this work adds to the current understanding of the gut-liver axis and development of MAFLD and will be of interest to many readers.

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#### **Reviewer #2 (Public review):**

The authors of this article investigated the impact of the host enzyme AOA<sup>H</sup> on the progression of MASLD in mice. To achieve this, they utilized whole-body Aoa<sup>H</sup><sup>-/-</sup> mice. The authors demonstrated that AOA<sup>H</sup> reduced LPS-induced lipid accumulation in the liver, probably by decreasing the expression and activation of SREBP1. In addition, AOA<sup>H</sup> reduced hepatic inflammation and minimized tissue damage.

However, this paper is descriptive without a clear mechanistic study. Another major limitation is the use of whole-body KO mice so the cellular source of the enzyme remains undefined. Moreover, since LPS-mediated SREBP1 regulation or LPS-mediated MASLD progression is already documented, the role of AOA<sup>H</sup> in SREBP1-dependent lipid accumulation and MASLD progression is largely expected.

Specific comments:

- (1) The overall human relevance of the current study remains unclear.
- (2) Is AOA<sup>H</sup> secreted from macrophages or other immune cells? Are there any other functions of AOA<sup>H</sup> within the cells?
- (3) Due to using whole-body KO mice, the role of AOA<sup>H</sup> in specific cell types was unclear in this study, which is one of the major limitations of this study. The authors should at least conduct in vitro experiments using a co-culture system of hepatocytes and Kupffer cells (or other immune cells) isolated from WT or Aoa<sup>H</sup><sup>-/-</sup> mice.
- (4) It has been well-known that intestinal tight junction permeability is increased by LPS or inflammatory cytokines. However, in Figure 3E, intestinal permeability is comparable between the groups in both diet groups. The authors should discuss more about this result. In

addition, intestinal junctional protein should be determined by Western blot and IHC (or IF) to further confirm this finding.

(5) In Figure 6, LPS i.g. Aoah<sup>-/-</sup> group is missing. This group should be included to better interpret the results.

(6) The term NAFLD has been suggested to be changed to MASLD as the novel nomenclature according to the guidelines of AASLD and EASL.

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