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Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease

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Abstract

Background: This study was designed to identify novel links between lipid species and disease progression in non-alcoholic fatty liver disease (NAFLD). Methods: We analyzed lipid species in the liver and plasma of db/db mice fed a choline-deficient L-amino acid-defined, high-fat diet (CDAHFD) using liquid chromatography/mass spectrometry (LC/MS). An in vitro experiment was performed using HepG2 cells stimulated with recombinant human TNFα or IL1β. The expression of steatosis-, inflammation-, and fibrosis-related genes were analyzed. Plasma samples from NAFLD patients were also analyzed by LC/MS. Results: The CDAHFD-fed db/db mice with hepatic steatosis, inflammation, mild fibrosis, obesity, and hypercholesterolemia displayed significantly higher hepatic and plasma levels of free adrenic acid ($p<0.05$). The accumulated adrenic acid in the CDAHFD-fed db/db mice was associated with increased expression of ELOVL2 and 5, and the suppression of the acyl-CoA oxidase 1 gene during peroxisomal β-oxidation. The pretreatment of HepG2 cells with adrenic acid enhanced their cytokine-induced cytokines and chemokines mRNA expression. In NAFLD patients, the group with the highest ALT levels exhibited higher plasma adrenic acid concentrations than the other ALT groups (p-value for trend: <0.001). Conclusion: Data obtained demonstrated that adrenic acid accumulation contributes to disease progression in NAFLD.

Keywords	Liquid chromatography/mass-spectrometry; non-alcoholic fatty liver disease; adrenic acid; chemokine; alanine aminotransferase.
Taxonomy	Liver Biochemistry, Metabolomics, Polyunsaturated Fatty Acid

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Submission Files Included in this PDF

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Response to Reviewers_Saut et al (for ABB)final.docx [Response to Reviewers]

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Prof. Shinya Toyokuni
Editor
Archives of Biochemistry and Biophysics

April 23, 2017

Subject: Submission of revised paper

Dear Professor Toyokuni,

Thank you for your email dated 22 April 2017 enclosing the reviewers' comments (Ref: YABBI_2017_86R1). We have carefully reviewed the comments and have revised the manuscript accordingly. Our responses are given in a point-by-point response. Changes to the manuscript are shown with yellow-highlighted letters.

All authors contribute to this research and are in agreement with its publication in *Archives of Biochemistry and Biophysics*. This content has not been submitted for publication elsewhere.

We hope the revised version is now suitable for publication of *Archives of Biochemistry and Biophysics*, and look forward to hearing from you.

Sincerely,

Shin Nishiumi

Response to Editor and Reviewers

Dear Editor and Reviewers,

Thank you very much for your constructive and helpful comments to improve the impact of our manuscript. According to your comments and suggestions, we have modified our manuscript as described below. The revision parts have been indicated with green-highlighted letters in our revised manuscript (The yellow-highlighted letters shows the first revision parts.).

Response to Reviewer 1:

Thank you very much for your review of our paper.

Comments from the editors and reviewers:
-Reviewer 1
Most of the criticisms have been solved in the revised manuscript.

Response: We are deeply grateful for agreeing with our revision.

Response to Reviewer 2:

Thank you for your comments on our paper. Our answers to your points are as follows.

Comments from the editors and reviewers:
-Reviewer 2
I didn't find the explanation about the identification of lipids in the Materials and methods section (page 10, line 536-549).

You should mention how you identified lipids.

Response: We apologize for incomplete response to your comments. In our revised manuscript, our previous article (**Ref 18; Matsubara et al., Journal of Chromatography B, 969 (2014) 199–204**) was newly cited. This article includes the list of the m/z value and retention time for the lipids of which the levels were examined in our study, and the information about the m/z value and retention time is required for the identification of lipids. The lipids evaluated by LC/ MS were putatively identified based on multiple reaction monitoring transitions (m/z value) and their retention times using an in-house library, which has the information about the targeted lipids including phospholipids, acylcarnitines, fatty acids, and bile acids (18). Phospholipids included in this library were verified on the basis of the precursor m/z and a head group-specific positive fragment (m/z 184.1) for phosphocholines or head group-specific neutral loss (NL 141) for phosphoethanolamines. The structural details of their constituent fatty acids were assigned on the basis of the numbers of C-atoms and double bonds in their negative fragments. Acylcarnitines included in this library were verified on the basis of the precursor m/z and specific positive fragment (m/z 85.05). Fatty acids and bile acids included in this library were identified using authentic chemical standards. These descriptions for the identification of lipids were added into the **Materials and methods** section (**page 10-11, line 542-570**).

Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease

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Abbreviations used**:**

ACOX (acyl Co-A oxidase), ALT (alanine aminotransferase), AC (acylcarnitine), BMI (body mass index), BSA (bovine serum albumin), CDAHFD (choline deficient L-amino acid defined high fat diet), COL1α1 (collagen type 1 alpha 1), CYP2E1 (cytochrome P450 family 2, subfamily e, polypeptide 1), CYP4A14 (cytochrome P450, family 4, subfamily a, polypeptide 14), ELOVL (elongase), FAS (fatty acid synthase), FATP (fatty acid transport protein), FFA (free fatty acid), GPx1 (glutathione peroxidase-1), IL (interleukin), IL1R (interleukin-1 receptor), LCAD (long chain acyl Co-A dehydrogenase), LC/MS (liquid chromatography/mass spectrometry), LPC (lysophosphatidylcholine), LPE (lysophosphatidylethanolamine), MCAD

(medium chain acyl Co-A dehydrogenase), MCP1 (monocyte chemoattractant protein-1), MIP1β (macrophage inflammatory protein-1 beta), MTTP (microsomal triglyceride transfer protein), NAFLD (non-alcoholic fatty liver disease), NASH (non-alcoholic steatohepatitis), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PUFA (polyunsaturated fatty acid), rh (recombinant human), RT-PCR (real-time reverse transcription polymerase chain reaction), SD (standard diet), TG (triglyceride), TGFβ1 (transforming growth factor-beta 1), TNFα (tumor necrosis factor-alpha), TNFR-1 (TNF receptor-1), UCP2 (uncoupling protein-2), USG (ultrasonography).

Abstract

Background: This study was designed to identify novel links between lipid species and disease progression in non-alcoholic fatty liver disease (NAFLD).

Methods: We analyzed lipid species in the liver and plasma of db/db mice fed a choline-deficient L-amino acid-defined, high-fat diet (CDAHFD) using liquid chromatography/mass spectrometry (LC/MS). An *in vitro* experiment was performed using HepG2 cells stimulated with recombinant human TNFα or IL1β. The expression of steatosis-, inflammation-, and fibrosis-related genes were analyzed. Plasma samples from NAFLD patients were also analyzed by LC/MS.

Results: The CDAHFD-fed db/db mice with hepatic steatosis, inflammation, mild fibrosis, obesity, and hypercholesterolemia displayed significantly higher hepatic and plasma levels of free adrenic acid ($p<0.05$). The accumulated adrenic acid in the CDAHFD-fed db/db mice was associated with increased expression of ELOVL2 and 5, and the suppression of the acyl-CoA oxidase 1 gene during peroxisomal β-oxidation. The pretreatment of HepG2 cells with adrenic acid enhanced their cytokine-induced cytokines and chemokines mRNA expression. In NAFLD patients, the group with the highest ALT levels exhibited higher plasma adrenic acid concentrations than the other ALT groups (p-value for trend: <0.001).

Conclusion: Data obtained demonstrated that adrenic acid accumulation contributes to disease progression in NAFLD.

Keywords

Liquid chromatography/mass-spectrometry; non-alcoholic fatty liver disease; adrenic acid; chemokine; alanine aminotransferase.

1. Introduction

Epidemiological studies have indicated that the prevalence of non-alcoholic fatty liver disease (NAFLD) is rising in both Western countries and other regions of the world (1,2). Along with the increasing incidence of obesity and its related metabolic diseases, it is predicted that NAFLD will become the major cause of chronic liver disease. Histologically, some NAFLD patients only exhibit steatotic changes in their livers and remain stable for a long period of time, whereas others develop marked liver inflammation and hepatocyte necrosis. The latter subset of patients, which are considered to have non-alcoholic steatohepatitis (NASH), are at increased risk of liver cirrhosis, hepatocellular carcinoma, and liver-related mortality (2–4). Therefore, the early identification of NASH is very important for preventing liver complications.

Disease progression in NAFLD is a complex process involving several factors, including lipid metabolism. Some theories have been postulated to explain how lipids can influence disease progression in NAFLD. The two-hit concept suggests that lipid accumulation serves as a 'first hit' that sensitizes the liver to various other hits, such as oxidative stress, pro-inflammatory cytokines, endotoxins, or hypoxia (5). For example, free cholesterol-sensitized hepatocytes undergo tumor necrosis factor (TNF)-induced apoptosis (6). On the other hand, the lipotoxicity concept suggests that a direct relationship exists between certain lipid species and liver damage. For example, free fatty acids (FFA), such as palmitic acid and lysophosphatidylcholines (LPC), can activate the intrinsic apoptotic pathway through endoplasmic reticulum stress and c-Jun NH2-terminal kinase activation (7,8). According to the multiple-hit concept, this lipotoxicity process together with genetic and dietary factors, adipose tissue dysfunction, and gut flora determine the extent of disease progression in NAFLD (9). On the other hand, ceramides have been also implicated in disease progression, as they promotes insulin resistance, which is a risk factor for NAFLD (10).

Despite extensive investigation of the molecular mechanisms responsible for lipid-related liver damage in NAFLD, the available quantitative data about the use of lipid profiles for differentiating steatohepatitis from simple steatosis are still inconclusive. Metabolomic-based lipid profiling (lipidomic) studies have shown that the fatty acid content of the liver was increased in NAFLD patients compared with controls, but no significant differences were detected between simple steatosis and steatohepatitis (11,12). Similar findings have also been obtained for free cholesterol, total LPC, and ceramide levels (11,13). On the other hand, Gorden et al. reported that the levels of several ceramide species were significantly increased in the plasma, but not the livers, of NASH patients, compared with those seen in simple steatosis patients (14). So far, lipidomic studies have suggested that at least 500 lipid species are present in plasma, and over 1,000 lipid species are found within cells (15,16). Hence, we hypothesized that there might be other lipid species that are pathologically related to NAFLD, and some of them might be useful for differentiating steatohepatitis from simple steatosis. One of the analytical methods used in lipidomic studies is liquid chromatography/mass spectrometry (LC/MS). LC/MS-based analysis makes it possible to profile lipid species accurately from a small minimally pre-treated sample within a short period of time (17). Thus, our aim is to identify novel links between lipid species and the progression of NAFLD using LC/MS.

In this study, we first examined the lipid profiles of genetically obese db/db mice and used a choline deficient-L-amino acid defined-high fat diet (CDAHFD) to induce steatohepatitis in the mice. The CDAHFD induced hepatic steatohepatitis and fibrosis, which are comparable to the pathology of human NASH, in db/db mice. Next, we tested our findings *in vitro* using HepG2 cells and finally confirmed them using samples from NAFLD patients.

2. Materials and methods

2.1. Ethical approval

All animal treatments in this study were approved by the institutional animal care and use committee and carried out according to the Kobe University animal experimentation regulations. The use of human blood samples was approved by the ethics committees at Kobe University Graduate School of Medicine and its related hospitals, and the analysis of these samples was carried out according to the guidelines of Kobe University Hospital. Informed consent was obtained from all human subjects.

2.2. Animal experiment

Six-week-old male db/db mice were randomly divided into two groups of five mice, which received different dietary formulas for 4 weeks. One group was fed a standard diet (SD; CE-2, CLEA Japan, Inc., Shizuoka, Japan) containing 3,449 kcal/kg. The other group was fed an L-amino-acid-defined, high-fat diet that contained 0.1% methionine but did not contain any choline (CDAHFD; #A06071313). The CDAHFD was purchased from Research Diet, Inc., (New Brunswick, NJ, USA) and contained 5,200 kcal/kg. We also used age-matched male C57BL/6J (B6) mice that were fed the SD for 4 weeks as a control group. The composition of each diet is shown in Supplementary Table S1. The body weights of the mice were recorded every week during the experimental period. On the final day of the experiment, the mice were sacrificed after overnight fasting, and their whole blood was collected by cardiac puncture using heparin as an anticoagulant. The heparinized blood was then centrifuged at 6,000 g for 10 min at 4^{o}C to obtain plasma samples. Before the liver was removed, it was perfused with distilled water and then weighed. A portion of the liver was fixed in 10% formalin buffer for histological evaluation. The plasma samples and remaining portion of the liver were kept at -80^{o}C until further use.

2.3. Liver histology

The formalin-fixed liver tissues were embedded in paraffin, sliced into thin sections, and then stained with standard hematoxylin and eosin (H&E). Hepatic fibrosis was assessed using the picrosirius red stain kit (Polysciences, Inc., USA).

2.4. Measurement of hepatic triglyceride levels

The lipids in the liver tissues of the mice were extracted according to a modified version of the Bligh and Dyer method. Briefly, the liver tissue was homogenized with 9 volumes of 1.15% (w/v) KCl, and aliquots (0.5 mL) of the homogenate were extracted with 2 mL of chloroform-methanol. To remove water-soluble substances, a 1/5 volume of 0.5% (w/v) NaCl was added. After being centrifuged at 1,500 g for 5 min, the resultant chloroform layer was evaporated and then dissolved in methanol supplemented with 10% Triton-X. The hepatic triglyceride (TG) level was quantified using a commercial kit (Wako Pure Chemical Industries, Tokyo, Japan) according to the manufacturer's instructions.

2.5. Measurement of plasma biochemistry

The plasma alanine transferase (ALT), TG, and total cholesterol levels of the mice were measured using commercial kits (Wako Pure Chemical Industries, Tokyo, Japan).

2.6. Fatty acid preparation

The fatty acid solution for the *in vitro* experiment was prepared by complexing 0.5 mM adrenic acid (Sigma Aldrich, USA) with 1.65% (*w/v*) bovine serum albumin (BSA; Wako Pure Chemical Industries, Tokyo, Japan) in serum-free medium, incubated at 37^{o}C for 1 hr, and then

subjected to filter sterilization. The fatty acid solution was freshly prepared for each experiment. The final molar ratio of the adrenic acid/BSA complex was 2:1.

2.7. **Cell culture conditions and treatment**

HepG2 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako Pure Chemical Industries, Tokyo, Japan) supplemented with 10% (*v/v*) fetal bovine serum (Invitrogen), 100 unit/ml penicillin and 100 μg/ml streptomycin. For the gene expression analysis, HepG2 cells (10^6 cells/well) were seeded onto 6-well plates (Falcon, Corning Inc., USA) and then allowed to rest for 24 hrs. Before the treatment assay, the cells were washed, and the medium was replaced with serum-free medium containing adrenic acid/BSA complex. Control cell cultures were incubated with serum-free medium containing BSA alone. After overnight incubation, the cells were treated with 10 ng/mL recombinant human (rh)TNFα (Reliatech GmbH, Germany) or 5 ng/mL interleukin (IL)1β (PeproTech, USA) in serum-free medium for a further 5 hrs. All cells were incubated in a humidified atmosphere at 37°C/5% CO_2.

2.8. **Quantitative real-time PCR**

Total RNA was extracted from the cultured cells and mouse liver tissue with TRIzol reagent (Invitrogen, Tokyo, Japan) and quantified using an ultraviolet spectrophotometer (ND-1000, NanoDrop, USA). Following the extraction procedure, 1 μg of RNA was treated with the RT^2 first strand kit (Qiaqen, USA) to eliminate contaminating genomic DNA and promote cDNA synthesis. Quantitative real-time PCR was then performed using the 7500 real-time polymerase chain reaction (PCR) system and power SYBR green reagent (Applied Biosystems). The sequences of the primers used for the PCR are listed in Supplementary Table S2. The PCR cycling protocol was as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 sec,

and 60°C for 1 min. Melting curve analysis was conducted after the amplification step to identify a specific PCR product. All mRNA expression levels were normalized to the mRNA expression level of mouse GAPDH or human β-actin. The relative expression levels of all genes were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.9. **Human samples**

After overnight fasting, venous blood samples were collected from 30 patients with NAFLD who were diagnosed during routine health checkups at Hotel Okura Clinic, Kobe, Japan, between April 2016 and September 2016. Blood samples from 10 healthy subjects (i.e., those with a body mass index (BMI) of <23 kg/m^2, normal liver function, and normal metabolic parameters) with normal liver ultrasonographic (USG) findings were also collected. All subjects underwent clinical, hematological, biochemical, and serological evaluations during the health checkup. For the fatty acid analysis, plasma was prepared and kept at -80°C. The diagnosis of NAFLD was based on USG findings: increased hepato-renal echo contrast with reduced penetration of the posterior segment of the right lobe and poor visualization of the hepatic vessels and diaphragm. A normal liver was defined as a liver with a homogenous parenchyma that exhibited similar or slightly higher echogenicity than the renal cortex and well-visualized hepatic vessels and diaphragm. The USG examinations were performed by experienced sonographers using a Siemens ACUSON S1000 (Siemens, USA), and the resultant images were reviewed independently by certified gastroenterologists. None of the patients had a history of alcoholism (>20 g alcohol/day), steatogenic drug use, viral hepatitis, autoimmunity, malignancy, hepatobiliary disease, or other chronic liver diseases.

The patients were divided into three groups based on their ALT levels: group 1: ALT <30 IU/L for males, ALT <20 IU/L for females; group 2: 30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females; group 3: ALT ≥60 IU/L for males, ≥40 IU/L for females.

2.10. **Liquid chromatography-mass spectrometry (LC/MS)-based lipid analysis**

The lipid species present in the mouse and human samples were analyzed by LC/MS. Frozen mouse liver tissue samples (~5 mg) were homogenized in 225 µL of methanol and 25 µL di-lauroyl phosphatidylcholine (the internal standard). Then, 80 µL of methanol and 10 µL of internal standard were added to the plasma samples (10 µL) and vortexed. Next, the samples were kept on ice for 10 min. After being centrifuged (16,000 g, 4°C, 5 min), the supernatants (25 µL) were collected and transferred to vials for analysis. The LC/MS analysis was performed on a Nexera LC system coupled to an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corp., Japan). The lipid species were separated using an octadecyl silylated silica column (InertSustain C18, 100 x 2.1 mm, 3 µm, GL Sciences, Tokyo, Japan) with a guard column (10 x 3 mm, 5 µm). The mobile phase for lipids consisted of A: 20 mM ammonium acetate in water and B: methanol. The flow rate was 0.4 mL/min, and the column oven temperature was 40°C. The gradient program for mobile phase B was as follows: 0 min, 80%; 13 min, 98%; 30 min, 98%; 30.1 min, 80%; and 35 min, 80%. The lipids evaluated by LC/ MS were putatively identified based on multiple reaction monitoring transitions and their retention times using an in-house library, which has the information about the targeted lipids including phospholipids, acylcarnitines, fatty acids, and bile acids (18). Phospholipids included in this library were verified on the basis of the precursor m/z and a head group-specific positive fragment (m/z 184.1) for phosphocholines or head group-specific neutral loss (NL 141) for phosphoethanolamines. The structural details of their constituent fatty acids were assigned on the

basis of the numbers of C-atoms and double bonds in their negative fragments. Acylcarnitines included in this library were verified on the basis of the precursor m/z and specific positive fragment (m/z 85.05). Fatty acids and bile acids included in this library were identified using authentic chemical standards. The peak areas under the curve of each lipid species were detected and then normalized to the internal standard. For the lipids extracted from liver tissue, after the data had been normalized, the relative amount was then calculated based on the weight of liver tissue prepared during the extraction process.

2.11. **Statistical analysis**

The significance of differences was analyzed using the Student's t-test or one-way ANOVA (SPSS version 22; SPSS, Inc., Chicago, IL, USA). For the human lipid data, comparisons of group means were conducted with one-way ANOVA and the Tukey honest significant difference test for across-group comparisons. A linear contrast analysis for ANOVA was also performed to test for linear trends in the mean values. P-values or p-values for trends of <0.05 were regarded as statistically significant. Unless stated otherwise, all data are presented as the mean ± standard error of mean (SEM).

3. **Results**

3.1. **Steatohepatitis and fibrosis were induced in the db/db mice fed the CDAHFD**

After 4 weeks' feeding, benign hepatic steatosis without inflammation was observed in the livers of the SD-fed db/db mice. On the other hand, severe steatosis, inflammatory foci, as well as hepatocyte ballooning, were seen in the livers of the CDAHFD-fed db/db mice (Figure 1). In agreement with this, the CDAHFD-fed db/db mice exhibited significantly higher hepatic mRNA levels of TNFα and CD68. Sirius red staining performed after 4 weeks' feeding showed mild

perisinusoidal fibrosis, which was indicative of an early stage of hepatic fibrosis, in the CDAHFD-fed db/db mice, but not the SD fed mice. In addition, the hepatic expression levels of transforming growth factor (TGF)β1 and collagen (COL)1α1 were significantly increased in the CDAHFD-fed db/db mice. The metabolic abnormalities seen in each group of mice are summarized in Table 1. Significant increases in body weight, liver weight, the hepatic TG level, the plasma total cholesterol level, and the ALT level were observed in the CDAHFD-fed db/db mice compared with the SD-fed db/db mice, while the plasma TG level did not differ significantly between the two groups (P=0.61).

3.2. **Oxidative stress-related gene expression**

To evaluate hepatic oxidative stress, we measured the hepatic mRNA levels of uncoupling protein-2 (UCP2) and glutathione peroxidase-1 (GPx1). Compared with the SD-fed db/db mice, the consumption of the CDAHFD diet was associated with the downregulation of hepatic GPx1 mRNA expression and the upregulation of hepatic UCP2 mRNA expression. The baseline hepatic GPx1 mRNA level of the SD-fed db/db mice was about three times higher than that of the B6 control mice, whereas their baseline hepatic UCP2 mRNA level was significantly lower than that seen in the B6 control mice (Table 1).

3.3. **Lipid metabolism-related gene expression**

The baseline hepatic fatty acid synthase (FAS) mRNA level of the db/db mice fed the SD was lower than that seen in the B6 control mice (Figure 2). Although the difference was not statistically significant, the CDAHFD-fed db/db mice displayed an even lower hepatic FAS mRNA level (P=0.061). The hepatic mRNA levels of medium- (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD), which are enzymes that are involved in the initial stages of

mitochondrial β-oxidation, did not differ significantly between the two db/db mouse groups. On the other hand, the CDAHFD-fed db/db mice exhibited lower hepatic mRNA levels of acyl-CoA oxidase (ACOX1), which is an enzyme that is involved in the initial stages of peroxisomal β-oxidation, than the SD-fed db/db mice. The SD-fed db/db mice displayed a significantly lower baseline hepatic mRNA level of microsomal TG transfer protein (MTTP) than the B6 control mice. Feeding the db/db mice the CDAHFD did not increase their MTTP mRNA expression. Compared with those seen in the B6 control mice, the baseline hepatic mRNA levels of cytochrome P450 2E1 (CYP2E1) and cytochrome P450, family 4, subfamily a, polypeptide 14 (CYP4A14) of the SD-fed db/db mice were significantly higher and lower, respectively. Feeding the db/db mice the CDAHFD did not increase the mRNA expression level of either enzyme. In the SD-fed db/db mice, the baseline hepatic mRNA level of fatty acid transport protein 2 (FATP2) was significantly lower than that seen in the B6 control mice, while the baseline hepatic mRNA level of FATP5 was increased. The hepatic mRNA levels of both FATP2 and 5 were significantly decreased in the CDAHFD-fed db/db mice.

3.4. **Lipid profiles of the liver and plasma**

In total, 236 species from 6 classes of lipids (phosphatidylcholines, PC; phosphatidylethanolamines, PE; LPC; lysophosphatidylethanolamines, LPE; FFA; acylcarnitines, AC) were detected in the liver and plasma samples (Supplementary Tables S3 & S4). Compared with those seen in the B6 control mice, the hepatic levels of 115 lipid species exhibited significant changes in the SD-fed db/db mice; i.e., the levels of 90 species were downregulated, and those of 25 were elevated. In a comparison between the CDAHFD-fed and SD-fed db/db mice, significant differences in the hepatic levels of 116 lipid species were detected; i.e., the hepatic levels of 73 and 43 species were downregulated and upregulated, respectively, in the

CDAHFD-fed mice. Of the hepatic lipid species that exhibited fold-change values of greater than or less than 2 between the SD-fed db/db and B6 control mice or between the CDAHFD-fed and SD-fed db/db mice, the 6 in each fold-change category whose intergroup differences were most significant are shown in Table 2.

In the analysis of the plasma samples, 130 out of 236 lipid species demonstrated significant differences in their plasma levels between the SD-fed db/db mice and the B6 control mice, and 98 of these species were downregulated in the SD-fed db/db mice. When we compared the plasma samples of the SD-fed db/db mice with those of the CDAHFD-fed db/db mice, we found that 139 lipid species exhibited significantly different levels between the two groups, with 43 lipid species demonstrating lower levels in the CDAHFD-fed db/db mice. Of the plasma lipid species that exhibited fold-change values of greater than or less than 2 between the SD-fed db/db and B6 control mice or between the CDAHFD-fed and SD-fed db/db mice, the 6 in each fold-change category whose intergroup differences were most significant are shown in Table 3.

In a detailed examination of the lipid profiles of the mice, we found that the plasma level of free eicosapentaenoic acid (20:5n3) was higher in the SD-fed db/db mice than in the B6 control mice. Similarly, we also found that the plasma and hepatic levels of phospholipids and lysophospholipids containing 20:5n3 were also increased in the SD-fed db/db mice. Interestingly, the hepatic level of free adrenic acid (22:4n6) was markedly higher in the CDAHFD-fed db/db mice than in the SD-fed db/db mice. In plasma, the difference in the adrenic acid level reached statistical significance ($p<0.001$). We also found that the concentrations of phospholipid species containing adrenic acid, such as PC or PE (40:4) (containing side chain 18:0/22:4), and LPC 22:4 (sn-1/sn-2), were also significantly increased. The hepatic and plasma levels of adrenic acid, which is an omega 6 polyunsaturated fatty acid (PUFA), exhibited greater differences between

the CDAHFD-fed db/db mice and the SD-fed db/db mice (8.6-fold higher vs. 1.6-fold higher in liver tissue, 5.3-fold higher vs. 2.2-fold higher in plasma) than those of well-known pro-inflammatory PUFA, such as arachidonic acid (Figure 3). Since the contribution of adrenic acid to NAFLD has not been examined in detail, we next focused on elucidating its potential role in the progression of the disease.

3.5. Increased mRNA expression of elongase 2 and 5 in the CDAHFD-fed db/db mice

First, in order to understand why the hepatic level of adrenic acid was markedly increased in the CDAHFD-fed db/db mice, the hepatic mRNA levels of ELOVL2 and 5 were examined using quantitative real-time PCR (Figure 4). Adrenic acid is produced via the elongation of arachidonic acid by ELOVL2 and 5. We detected significantly higher hepatic mRNA levels of ELOVL2 and 5 in the CDAHFD-fed db/db mice than in the SD-fed db/db mice. However, the SD-fed db/db mice displayed significantly lower basal hepatic expression levels of ELOVL2 and 5 than the B6 control mice.

3.6. Enhancement of proinflammatory cytokine-induced mRNA expression in adrenic acid-pretreated HepG2 cells

To confirm the role of adrenic acid in inflammation, we first pretreated HepG2 cells with 0.5 mM of adrenic acid before stimulating them with rhTNFα or IL1β (Figures 5 & 6). The concentration range of total adrenic acid in plasma of healthy person was from the trace amount to 158.4 μM, as reported previously (19). In the pathological state, as shown by our experimental findings, the fatty acid could increase two to five times fold. Based on this consideration, 0.5 mM of adrenic acid are within the pathological range, and then was used for *in vitro* experiment. Compared with TNFα treatment alone, the HepG2 cells that were pretreated with adrenic acid

and then treated with TNFα expressed higher mRNA levels of TNFα, IL8, macrophage inflammatory protein 1β (MIP1β), and monocyte chemoattractant protein 1 (MCP1). Under IL1β stimulation, the HepG2 cells that were pretreated with adrenic acid and then treated with IL1β expressed higher mRNA levels of TNFα, IL8, MIP1β, and TGFβ1, but lower MCP1 mRNA levels.

3.7. **Changes in the expression of IL-1 receptors in adrenic acid-pretreated HepG2 cells**

We next examined whether adrenic acid induced cytokine receptor expression (Figure 7). The mRNA level of TNF receptor type I (TNFR-1) was not significantly upregulated in adrenic acid-pretreated HepG2 cells that were subsequently treated with rhTNFα. In contrast, the mRNA expression of the type I IL-1 receptor fell significantly, and that of the type II IL-1 receptor increased significantly after the cells were treated with rhIL1β

3.8. **Association between the plasma levels of adrenic acid and ALT in human NAFLD**

Finally, we focused on measuring the relative abundance of FFA in the plasma of NAFLD patients and comparing these values among three groups based on the NAFLD patients' ALT levels. Overall, the NAFLD patients were older, had higher BMI, and were more dyslipidemic than the normal liver patients. There were no significant differences in age, gender, BMI, lipid or glucose parameters, or liver function among the NAFLD patients (Table 4). ANOVA showed that the mean plasma levels of palmitic acid (16:0), stearic acid (18:0), arachidic acid (20:0), erucic acid (22:1n9), eicosadienoic acid (20:2n6), adrenic acid (22:4n6), docosapentaenoic acid (22:5n6), stearidonic acid (18:4n3), eicosapentaenoic acid (20:5n3), and docosahexaenoic acid (22:6n3) differed significantly between the groups (Table 5). All of these fatty acids, except for 20:0 and 22:1n9, exhibited significant positive trends across the groups. Erucic acid (22:1n9)

displayed a decreasing trend as the ALT increased, but it did not reach the prespecified 0.05 significance level for linear trends (p=0.057). Post-hoc analysis showed that only adrenic acid (22:4n6) and docosapentaenoic acid (22:5n6) displayed significantly higher mean values in group 3 (ALT ≥60 IU/L for males; ALT ≥40 IU/L for females) than in the normal liver group or the group 1 (ALT <30 IU/L for males, <20 IU/L for females) or group 2 (30 ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females) NAFLD patients.

4. **Discussion**

The main findings of this study are as follows: (i) the plasma and hepatic levels of free adrenic acid were markedly increased in a mouse model of steatohepatitis, but not in a mouse model of simple steatosis; (ii) the increased hepatic levels of adrenic acid were due to increased endogenous synthesis and decreased catabolism in peroxisomes; (iii) pretreatment with adrenic acid was associated with enhanced mRNA expression of proinflammatory molecules in cytokine-stimulated hepatocytes; (iv) high ALT levels (≥60 IU/L in males, ≥ 40 IU/L in females) were associated with higher plasma adrenic acid levels in NAFLD patients.

A previous study showed that the CDAHFD induced fibrotic steatohepatitis in the livers of C57BL/6J mice (20). In our study, the CDAHFD also induced steatohepatitis and fibrosis in db/db mice, as confirmed histologically and through gene expression analysis. However, in contrast with the methionine choline-deficient diet, the CDAHFD induced body weight gains in the db/db mice. In addition, the total cholesterol level of the CDAHFD-fed mice also rose. Therefore, this group of mice exhibits a NASH-like phenotype combined with obesity and hypercholesterolemia, two features which are commonly found in human NAFLD. NASH is also associated with oxidative stress due to an imbalance between the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and antioxidant activity, such as that of GPx.

Oxidative stress can cause impaired mitochondrial oxidative phosphorylation, leading to the upregulation of the expression of UCP2, an uncoupling protein (21,22). In our study, we did not directly measure H_2O_2 production, but the increased expression of GPx1 seen in the SD-fed db/db mice suggests that hepatic steatosis is associated with increased ROS production. Thus, increased expression of GPx1 would have helped the SD-fed db/db mice to keep their redox state in balance, and therefore, UCP2 mRNA expression was not markedly induced in these mice. On the other hand, the CDAHFD diet suppressed GPx1 mRNA expression, which might have tilted the redox state of the CDAHFD-fed mice toward ROS production. As an adaptive response, UCP2 mRNA expression was upregulated and might have caused proton leakage across the inner mitochondrial membrane and impaired ATP production. Overall, this process could be associated with inflammation, liver damage, and increased plasma ALT levels in CDAHFD-fed db/db mice.

Hepatic steatosis can be caused by the impairment of several metabolic pathways, such as lipid uptake (FATP2 & 5), de novo lipogenesis (FAS), mitochondrial β-oxidation (MCAD, LCAD), peroxisomal β-oxidation (ACOX1), microsomal ω-oxidation (CYP2E1, CYP4A14), and lipid export (MTTP). The results of the present gene expression analysis indicated that the impaired secretion of TG-rich very low density lipoproteins and increased fatty acid uptake in the liver were associated with hepatic steatosis in our simple steatosis model. On the other hand, additional impairment of peroxisomal β-oxidation without further upregulation of microsomal ω-oxidation contributed to the severe steatosis observed in the livers of our steatohepatitis model. The absence of a further upregulation of CYP2E1 and CYP4A14 mRNA expression in our steatohepatitis model might have been due to inflammation. Previous studies have shown that

hepatic CYP2E1 and CYP4A14 expression were suppressed by inflammatory cytokines, such as TNFα (23,24).

Lipid analysis of NAFLD has consistently shown an increase in the n6/n3 ratio of PUFA, mainly due to the depletion of n3 species. However, the exact alterations in the level of each n6 PUFA remain to be elucidated. Yamada et al. showed that the levels of n6 PUFA, such as linoleic acid (18:2n6) and docosadienoic acid (22:2n6), were higher in liver tissue from patients with simple steatosis or steatohepatitis than in control liver tissue (12), while others found that arachidonic acid exhibited relatively low levels within several lipid classes (11,25,26). In the plasma of patients with simple steatosis and steatohepatitis, the concentration of linoleic acid was decreased while those of gamma-linolenic acid (18:3n6) and dihomo-gamma-linolenic acid (20:3n6) were increased (27). In our study, the CDAHFD-fed db/db mice demonstrated significantly higher levels of eicosadienoic acid (20:2n6), arachidonic acid (20:4n6), and adrenic acid (22:4n6) than the SD-fed db/db mice, and adrenic acid (22:4n6) exhibited the biggest differences between the groups. Furthermore, the levels of some phospholipid species containing adrenic acid were also significantly increased. Taken together, these data suggest that n6 PUFA synthesis was increased in the livers of our steatohepatitis model, which resulted in adrenic acid enrichment. Increased plasma total adrenic acid levels, which include both the esterified type and the unesterified free type of adrenic acid, have been detected in NASH patients (28). The plasma concentrations of phospholipids containing adrenic acid were also increased in NASH-associated hepatocellular carcinoma patients (29). In our study, the plasma level of free adrenic acid was increased in mice with steatohepatitis and NAFLD patients. The abovementioned studies and our results strongly suggest that adrenic acid contributes to steatohepatitis.

Endogenous adrenic acid is produced via the elongation of arachidonic acid by ELOVL2 or 5, and it is predominantly oxidized or chain-shortened in peroxisomes (30,31). ACOX catalyzes the first step in peroxisomal β-oxidation. In mammals, there at least three isoforms of ACOX: ACOX1, ACOX2, and ACOX3 (32), and adrenic acid is a substrate for ACOX1 (33). Compared with those seen in the SD-fed db/db mice, the ACOX1 mRNA level was decreased in the CDAHFD-fed db/db mice, but the expression levels of ELOVL2 and 5 were increased. These data suggest that the higher hepatic and plasma levels of adrenic acid seen in the steatohepatitis model were caused by both increased endogenous synthesis and decreased catabolism in the hepatic peroxisomes.

Peroxisomes are extramitochondrial organelles that participate in fatty acid oxidation in the liver, as does the cytochrome P450-containing endoplasmic reticulum. An early study detected greater peroxisome-based β-oxidation in genetically obese mice than in lean mice (34). However, the impairment of peroxisome β-oxidation might be a key factor in the progression of simple steatosis to steatohepatitis. Mice deficient in ACOX genes were found to develop severe hepatic steatosis from a young age. By 5 months of age, the ACOX-deficient mice displayed hepatocyte apoptosis and lipogranuloma formation, which subsequently progressed to hepatocellular carcinoma (35). Meanwhile, Mitsuyoshi et al. detected lower hepatic expression of ACOX in NASH patients than in patients with simple steatosis and demonstrated that hepatic ACOX expression decreased further as the disease progressed (36). However, adrenic acid levels were not investigated in these studies.

ELOVL2 and 5 are very long chain fatty acid elongase enzymes. Both are expressed in the liver, where C20 and C22 PUFA are elongated by ELOVL2, while ELOVL5 elongates C18 and C20 PUFA (37). There is a lack of convincing evidence regarding the promotion of NASH by

ELOVL2 or 5, but lower hepatic expression of ELOVL5 has been reported in mice with high fat diet-induced fatty liver (38). A study by Moon et al. suggested that the deletion of ELOVL5 increased the nuclear abundance of sterol regulatory element-binding transcription factor 1 and hepatic steatosis by depleting docosahexaenoic acid (DHA, 22:6n3) (39). The deletion of ELOVL2 also causes the DHA depletion and upregulated expression of lipogenic genes, albeit without leading to hepatic steatosis (40). In our study, we also found a trend towards lower DHA levels in the steatotic livers of the SD-fed db/db mice compared with the B6 control mice. Although the CDAHFD-fed db/db mice exhibited higher ELOVL2 and 5 expression, the relative abundance of DHA in the CDAHFD-fed db/db mice did not differ significantly from that seen in the SD-fed db/db mice. This might have been due to impaired peroxisomal β-oxidation, as DHA is generated via the oxidation of tetracosahexaenoic acid (24:6n3) in peroxisomes.

In our study, the db/db mice fed the SD did not instantly develop hepatic steatohepatitis despite suffering from marked obesity compared with the B6 control mice. While this finding is in agreement with a previous study (41), there is still no firm explanation for it. Our lipid analysis suggests that the above finding could be due to the increased abundance of eicosapentaenoic acid (20:5n3), an antiinflammatory lipid. db/db mice have a functional defect in the long form leptin receptor, which impairs satiety, and hence, leptin can not inhibit food intake (42). Although the SD-fed db/db mice consumed a similar type of diet to the B6 control mice and exhibited lower basal expression of ELOVL2 and 5, this might have resulted in the saturation of ELOVL2 and 5 reactions, leading to the accumulation of eicosapentaenoic acid (20:5n3).

The presence of inflammation can predict the progression of NASH to advanced fibrosis (43). Therefore, we investigated the influence of adrenic acid on proinflammatory gene expression using HepG2 cells (a hepatocyte model). In this study, the pretreatment of HepG2 cells with

adrenic acid augmented autocrine TNFα expression. Elevated TNFα levels have been detected in the blood and livers of NAFLD patients (44,45). We also found that adrenic acid pretreatment enhanced the mRNA expression levels of TNFα-induced chemokines, such as IL8, MIP1β, and MCP1. The main function of chemokines is to attract immune cells to inflammatory sites. Chemokines themselves have been implicated in several inflammatory diseases, including those with metabolic components, such as atherosclerosis, obesity, and diabetes (46). In human NAFLD, Bertola et al. detected increased hepatic mRNA expression of several chemokines, including IL8, MIP1β, and MCP1 (47). Accordingly, the liver pathology of NASH usually involves the infiltration of a mixture of inflammatory cells, such as neutrophils, monocytes, and macrophages (48). Elevated IL1β levels have also been detected in an animal model of NASH and human NASH (49,50). Therefore, we also evaluated gene expression under IL1β treatment. Compared with TNFα, IL1β is a more potent inducer of TNFα, IL8, MIP1β, and MCP1 expression in HepG2 cells. The mRNA levels of all of these chemokines, except for MCP1, were upregulated in HepG2 cells and further enhanced in adrenic acid-pretreated HepG2 cells. This suggests that the upregulating effect of adrenic acid on MCP1 mRNA expression during inflammation is cytokine-specific.

The inflammatory activities of TNFα and IL1β are mediated by type 1 TNF receptors (TNFR-1) and type 1 IL-1 receptors (IL1R-1), respectively. On the other hand, type 2 IL-1 receptors (IL1R-2) act as "decoy receptors" and inhibit IL1β activity (51). Gene expression analysis of these receptors in adrenic acid-pretreated HepG2 cells indicated that: (i) negative regulation by IL1R-2 inhibits IL1β-induced inflammation, and (ii) adrenic acid might modify TNFα or IL1β signaling pathways at the post-receptor level. The induction of chemokine expression by TNFα or IL1β is dependent on the activation of NF-kappaB and activator protein-

1 (AP-1). It is possible that the release of free adrenic acid (or related metabolites) after TNFα or IL1β stimulation leads to sustained activation of NF-kappaB or AP-1. Therefore, further studies are needed to investigate the effects of free adrenic acid on NF-kappaB and the AP-1 signaling pathway.

In our *in vitro* study, neither TNFα nor IL1β induced TGFβ1 mRNA expression in HepG2 cells. However, it was significantly increased in adrenic acid-pretreated HepG2 cells after IL1β treatment. It is possible that the more intense inflammation seen in adrenic acid-pretreated HepG2 cells after IL1β treatment is accompanied by hepatocyte damage and increased oxidative stress, which subsequently stimulates TGFβ1 mRNA expression. Hepatocyte damage-induced TGFβ1 release could enhance stellate cell activation, leading to extracellular matrix synthesis and hepatic fibrosis (52,53). Besides TGFβ1, several pieces of evidence also suggest that chemokines themselves can act as profibrogenic mediators. Liver stellate cells express C-C chemokine receptor type 2 (CCR2) (54) and CCR5 (55), which are receptors for MCP1 and MIP1β, respectively. Stellate cells can be activated by IL8 (56), while MCP1 and MIP1β can induce their migration (54,57,58). These findings suggest that adrenic acid might also influence the progression of fibrosis by upregulating the expression of profibrotic chemokines in hepatocytes.

In our experimental model, the level of LPC-containing adrenic acid was also increased, suggesting its potential role in inflammation. Previous studies have shown LPC as a pro-inflammatory metabolite by inducing lipoapoptosis and the release of inflammatory extracellular vesicles (8,59). These evaluations, however, was limited to the LPC-containing saturated fatty acids. Therefore, a future study is needed to extend previous findings to LPC-containing adrenic acid. A higher relative abundance of adrenic acid raises a hypothesis that adrenic acid has a

larger activity or a stronger pro-inflammatory lipid than arachidonic acid. However, a comparative study between these two fatty acids is problematic, because arachidonic acid itself can be elongated to adrenic acid by ELOVLs. A future study using an unmetabolized analog of arachidonic acid should clarify this hypothesis.

To confirm the relationship between increased adrenic acid production and liver inflammation in the clinical setting, we examined the association between plasma adrenic acid levels and ALT levels in NAFLD patients. A previous study by Suzuki et al. showed that changes in the serum ALT levels of NASH patients were correlated with liver inflammation, in both univariate and multivariate analyses (60). Therefore, we divided our patients into three groups based on their ALT levels (different cut-off values were employed for males and females). In our data analysis, we found that the mean plasma adrenic acid level was higher in group 3 (ALT ≥60 IU/L for males, ≥40 IU/L for females) than in group 1 (ALT <30 IU/L for males, <20 IU/L for females) and group 2 (30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females). These intergroup differences and the associated linear trend were statistically significant. Overall, these findings support our *in vitro* results. We also obtained a similar finding with regard to the plasma level of docosapentaenoic acid (22:5n6) in our human data set. Since the formation of docosapentenoic acid (22:5n6) involves β-oxidation in peroxisomes, this might indicate that our patients had mild disease that did not involve significant changes in peroxisome β-oxidation, as shown by the mouse model. A positive correlation between serum FFA levels and ALT was detected in NAFLD in a previous study (61). Our results provide more information by showing which fatty acids are associated with increased ALT levels in NAFLD. The limitations of our human sample analysis include the small number of patients, the lack of histological examinations, and the fact that it only included patients from one medical center.

In summary, the combination of increased PUFA and elongase expression and impaired peroxisomal β-oxidation cause the accumulation of adrenic acid in experimental steatohepatitis, but not in simple steatosis. Under inflammatory conditions, adrenic acid might exacerbate inflammation by enhancing the expression of chemokine genes in hepatocytes. Increased plasma adrenic acid levels are associated with high ALT levels in NAFLD patients. Taken together, adrenic acid accumulation contributes to disease progression in NAFLD. A further study is needed to determine whether adrenic acid measurements could be used as a diagnostic biomarker of steatohepatitis in NAFLD.

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Disclosure Statement

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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Table 1. Metabolic features and hepatic mRNA expression levels of db/db mice fed the SD or CDAHFD at 4 weeks

	C57BL/6J	db/db	
Diet	SD	SD	CDAHFD
Body weight (g)			
Initial	21.0 ± 0.3	37.4 ± 0.5*	36.6 ± 1.1
Final	25.6 ± 0.2	42.5 ± 1.3*	51.0 ± 2.6#
Liver weight (g)	2.0 ± 0.06	3.5 ± 0.1*	5.05 ± 0.7#
Hepatic TG (mg/g)	12.7 ± 0.7	26.4 ± 2.7*	69.3 ± 9.3#
Plasma TG (mg/dl)	27.5 ± 4.8	120.4 ± 14.7*	111.3 ± 8.5
Plasma total cholesterol (mg/dl)	89.2 ± 1.7	128.1 ± 6.3*	227.4 ± 9.6#
Plasma ALT (IU/L)	12.3 ± 1.4	18.9 ± 4.3	110.7 ± 8.6#
Hepatic mRNA expression			
TNFα	1.0 ± 0.3	0.8 ± 0.2	2.9 ± 0.5#
CD68	1.0 ± 0.1	0.3 ± 0.03*	1.7 ± 0.3#
TGFβ1	1.0 ± 0.02	1.1 ± 0.2	2.0 ± 0.1#
COL1α1	1.0 ± 0.2	0.5 ± 0.1*	4.2 ± 0.6#
GPx1	1.0 ± 0.03	2.9 ± 0.3*	1.0 ± 0.15#
UCP2	1.0 ± 0.1	0.5 ± 0.08*	1.1 ± 0.17#

SD, standard diet; CDAHFD, choline deficient L-amino acid defined high fat diet; TG, triglyceride; ALT, alanine aminotransferase; TNFα, tumor necrosis factor-alpha; TGFβ1, transforming growth factor-beta 1; COL1α1, collagen type 1, alpha 1; GPx1, glutathione peroxidase-1; UCP2, uncoupling protein-2;

Hepatic mRNA levels were analyzed by RT-PCR. GAPDH was used as an endogenous control. Data are shown as mean ± SEM (n=5) values. *$p<0.05$ compared with the C57BL/6J, SD mice; # $p<0.05$ compared with the db/db, SD mice

Table 2. The six lipid species in the <2 and >2 fold-change categories that exhibited the most significant intergroup differences in their hepatic levels

Species	Fold (SS/CT)	P-value	Species	Fold (SH/SS)	P-value
PE (16:1/20:5)	4.52	0.0051	FA 22:4n6	8.67	0.0045
LPC 20:5 (sn-1)	4.23	0.0274	LPC 22:4 (sn-1)	5.72	0.0026
LPE 20:5 (sn-1)	3.49	0.0076	PC (20:1/20:3_18:0/22:4)	4.99	<0.001
LPC 20:3 (sn-1)	3.41	0.0025	PE (16:0p/20:4)	3.5	<0.001
PC (18:1/20:2_18:0/20:3)	3.05	0.0028	PC (18:1e/20:3)	2.85	0.0092
PE (16:0/20:5)	3.03	<0.001	PE (18:0p/20:4)	2.75	<0.001
PE (20:0e/18:1)	0.13	0.0205	PE (16:1/20:5)	0.06	0.0025
PE (18:0/22:4_20:0/20:4)	0.15	0.0137	LPE 20:5 (sn-1)	0.1	0.0037
PE (19:0/20:4)	0.17	<0.001	PC (15:0/20:5)	0.13	0.0070
PE (20:1/22:6)	0.22	0.0014	LPC 20:5 (sn-1)	0.16	0.0203
PC (18:1e/22:5)	0.26	0.0832	PE (18:0/20:5)	0.19	<0.001
PC (18:0p/18:1_18:1e/18:1)	0.26	0.0017	PC (16:1/20:5)	0.19	0.0140

CT, controls (C57BL/6J mice fed the SD); SS, simple steatosis (db/db mice fed the SD); SH, steatohepatitis (db/db mice fed the CDAHFD); PE, phosphatidylethanolamines; PC, phosphatidylcholines; LPC; lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; FA, fatty acids

P-values were obtained using the Student's t-test.

Table 3. The six lipid species in the <2 and >2 fold-change categories that exhibited the most significant intergroup differences in their plasma levels

Species	Fold (SS/CT)	P-value	Species	Fold (SH/SS)	P-value
PC (16:0/22:2)	6.52	<0.001	PC (20:1/20:3_18:0/22:4)	10.99	<0.001
LPE 20:5 (sn-2)	6.37	0.0014	LPC 22:4 (sn-1)	9.36	<0.001
LPE 20:5 (sn-1)	3.92	0.0199	LPC 22:4 (sn-2)	7.94	<0.001
FA 20:5n3	3.15	<0.001	PE (18:0/22:4_20:0/20:4)	7.32	0.0077
LPC 20:5 (sn-2)	2.8	0.0231	PC (18:1e/20:5)	6.62	0.0117
PC (18:1/20:3)	2.7	<0.001	FA 22:4n6	5.33	<0.001
PC (18:1e/20:5)	0.12	<0.001	LPE 20:5 (sn-1)	0.18	0.0152
PE (20:0e/20:4_18:0e/22:4)	0.23	0.0243	PE (16:0p/20:5)	0.18	0.0740
PE (17:0/22:6)	0.23	<0.001	LPC 20:5 (sn-2)	0.23	0.0128
PE (16:0p/20:4)	0.26	<0.001	PE (16:1/20:5)	0.24	0.0157
PE (17:0/20:4)	0.27	0.0012	PE (18:1p/20:3_16:0p/22:4)	0.24	<0.001
PE (16:0/22:4)	0.28	0.0274	PE (18:0/20:5)	0.25	<0.001

CT, control (C57BL/6J mice fed the SD); SS, simple steatosis (db/db mice fed the SD; SH, steatohepatitis (db/db mice fed the CDAHFD). PE, phosphatidylethanolamines; PC, phosphatidylcholines; LPC; lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; FA, fatty acids

P-values were obtained using the Student's t-test

Table 4. Characteristics of USG-diagnosed NAFLD patients according to their ALT levels

	Normal liver	NAFLD		
		Group 1	Group 2	Group 3
	(n=10)	(n=10)	(n=10)	(n=10)
Sex (F/M)	5/5	6/4	5/5	6/4
Age (years)	39.5 ± 1.3	51.7 ± 2.2*	47.2 ± 1.9*	47.8 ± 1.8*
BMI (kg/m^2)	19.6 ± 0.5	29.1 ± 0.8*	27.0 ± 0.5*	28.4 ± 1.2*
AST (IU/L)	19.0 ± 1.3	18.1 ± 1.3	23.9 ± 0.9	50.3 ± 6.6*#
ALT (IU/L)	17.2 ± 2.0	17.8 ± 1.6	35.2 ± 1.4	77.1 ± 13.5*#
Albumin (g/dl)	4.5 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
Total bilirubin (mg/dl)	0.8 ± 0.1	0.8 ± 0.05	0.8 ± 0.07	0.7 ± 0.06
Platelets (x10^4/mm^3)	22.2 ± 0.8	25 ± 1.3	27.4 ± 2.8	26.2 ± 1.6
TG (mg/dl)	74.1 ± 12.4	122.7 ± 15.3	155.6 ± 25	199.4 ± 29.3*
Total cholesterol (mg/dl)	181.5 ± 6.3	213.8 ± 10.3	221.5 ± 9.2*	230.5 ± 12.1*
LDL (mg/dl)	102.1 ± 6.2	138.1 ± 8.7*	142.6 ± 6.4*	146.4 ± 10.6*
HDL (mg/dl)	72.1 ± 4.2	59.8 ± 4.9	58 ± 3.9	56.1 ± 3.1*
Fasting glucose (mg/dl)	93.5 ± 3.4	98.4 ± 2.3	100 ± 2.6	99.5 ± 2.0
HbA1c (%)	5.4 ± 0.1	5.7 ± 0.06*	5.6 ± 0.07	5.8 ± 0.1*

Data are shown as the mean ± SEM. Group 1: ALT <30 IU/L for males, ALT <20 IU/L for females; group 2: 30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females; group 3: ALT ≥60 IU/L for males, ≥40 IU/L for females

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine transaminase; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin

* $p<0.05$ compared with the normal liver group; # $p<0.05$ compared with groups 1 and 2

Table 5. Relative abundance of plasma FFA in NAFLD patients based on their ALT levels

	Normal liver		NAFLD						P-value	P-value for trend
			Group 1		Group 2		Group 3			
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM		
Saturated fatty acids										
12:0	0.077	0.027	0.086	0.008	0.07	0.006	0.074	0.006	0.488	0.452
14:0	0.372	0.052	0.388	0.038	0.386	0.041	0.49	0.062	0.319	0.121
16:0	13.407^{b}	1.342	16.706^{ab}	1.17	16.161^{ab}	1.349	20.084^{a}	1.671	**0.017**	**0.004**
17:0	0.078	0.011	0.071	0.006	0.083	0.01	0.082	0.007	0.768	0.605
18:0	7.731^{b}	0.691	8.617^{ab}	0.391	8.175^{b}	0.665	10.542^{a}	0.591	**0.011**	**0.005**
20:0	0.075^{a}	0.009	0.062^{ab}	0.003	0.049^{b}	0.002	0.062^{ab}	0.004	**0.013**	**0.033**
21:0	0.023	0.003	0.019	0.002	0.015	0.002	0.024	0.004	0.074	0.777
22:0	0.114	0.015	0.09	0.006	0.087	0.005	0.084	0.007	0.104	**0.032**
23:0	0.048	0.007	0.042	0.005	0.037	0.004	0.046	0.006	0.525	0.622
Monounsaturated fatty acids										
14:1n5	0.124	0.024	0.114	0.013	0.118	0.009	0.15	0.012	0.41	0.279
16:1n7	1.512	0.328	1.645	0.24	1.518	0.139	2.126	0.278	0.295	0.144
17:1n7	0.235	0.036	0.241	0.024	0.259	0.019	0.307	0.031	0.283	0.075
20:1n9	0.619	0.093	0.606	0.095	0.569	0.054	0.701	0.086	0.721	0.581
22:1n9	0.200^{a}	0.029	0.151^{ab}	0.026	0.075^{b}	0.018	0.142^{ab}	0.038	**0.031**	0.057
24:1n9	0.166	0.019	0.180	0.024	0.145	0.01	0.163	0.017	0.598	0.585
Polyunsaturated fatty acids										

18:2n6	6.974	0.927	7.747	0.786	7.422	0.645	8.59	0.717	0.516	0.201
20:2n6	0.226[b]	0.03	0.249[ab]	0.023	0.274[ab]	0.021	0.342[a]	0.036	**0.039**	**0.006**
20:4n6	0.466	0.107	0.524	0.037	0.513	0.042	0.664	0.05	0.182	0.054
22:4n6	0.065[b]	0.005	0.104[b]	0.009	0.103[b]	0.015	0.147[a]	0.01	**<0.001**	**<0.001**
22:5n6	0.215[b]	0.019	0.306[b]	0.024	0.326[b]	0.035	0.479[a]	0.046	**<0.001**	**<0.001**
18:4n3	0.016[b]	0.003	0.027[ab]	0.005	0.018[b]	0.002	0.039[a]	0.007	**0.006**	**0.009**
20:5n3	0.048[b]	0.005	0.076[ab]	0.012	0.075[ab]	0.011	0.113[a]	0.02	**0.013**	**0.002**
22:6n3	0.314[b]	0.035	0.412[b]	0.052	0.461[ab]	0.048	0.634[a]	0.079	**0.003**	**<0.001**

SEM, standard error of the mean; Group 1: ALT <30 IU/L for males, ALT < 20 IU/L for females; group 2: 30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females; group 3: ALT ≥60 IU/L for males, ≥40 IU/L for females. P-value, significance level for comparisons between groups; P-value for trend, significance level for the linear trend across the groups; Groups displaying different letters are significantly different (Tukey HSD test, $p<0.05$).

Figure legends

Figure 1. Representative liver histopathology of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

Liver sections were stained with H&E and sirius red (x200, scale bar: 100 um). The black arrow indicates an inflammatory focus, and the inset shows hepatocyte ballooning.

Figure 2. Relative expression levels of steatosis-related genes in the livers of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

Relative expression levels were determined using RT-PCR. GAPDH was used as the endogenous control. The mean expression level in the B6 mice fed the SD was set at 1. Data are shown as the mean ± SEM (n=5). * $p<0.05$ compared with the B6, SD mice; # $p<0.05$ compared with the db/db, SD mice

Figure 3. Relative abundance of free polyunsaturated fatty acids in the livers (A) and plasma (B) of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or the choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

The relative abundance of each PUFA was measured as described in the Methods section. The mean level seen in the B6 mice fed the SD was set at 1. Data are shown as the mean ± SEM (n=5). * $p<0.05$ compared with the B6, SD mice. # $p<0.05$ compared with the db/db, SD mice

Figure 4. Relative expression levels of ELOVL2 and ELOVL5 in the livers of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

Relative expression levels were determined using RT-PCR. GAPDH was used as an endogenous control. The mean expression level of the B6 mice fed the SD was set at 1. Data are shown as the mean ± SEM (n=5). * $p<0.05$ compared with the B6, SD mice; # $p<0.05$ compared with the db/db, SD mice

Figure 5. Relative expression levels of proinflammatory genes in adrenic acid (ADA)-pretreated HepG2 that had been stimulated with rhTNFα

Relative expression levels were determined using RT-PCR. The mRNA levels of TNFα, IL8, and MIP1β were normalized to natural logarithm values. All bars indicate the difference compared with the untreated cells, except for MCP1. No MCP1 mRNA expression was detected in the untreated cells, so the mean expression level of MCP1 in the rhTNFα-treated HepG2 cells was set at 1. β-actin was used as an endogenous control. Data are shown as the mean ± SEM (n=3). *$p<0.05$ compared with the rhTNFα-treated HepG2 cells; n.s.: not significant

Figure 6. Relative expression levels of proinflammatory genes in adrenic acid (ADA)-pretreated HepG2 that had been stimulated with rhIL1β

Relative expression levels were determined using RT-PCR. The mRNA levels of TNFα, IL8, and MIP1β were normalized to natural logarithm values. All bars indicate the difference compared with the untreated cells, except for MCP1. No MCP1 mRNA expression was detected in the untreated cells, so the mean expression of MCP1 in the rhIL1β-treated HepG2 cells was

set at 1. β-actin was used as an endogenous control. Data are shown as the mean ± SEM (n=3). *p<0.05 compared with the rhIL1β-treated HepG2 cells

Figure 7. Relative expression levels of TNFR-1(A), IL1R-1, and IL1R-2 (B) in adrenic acid (ADA)-pretreated HepG2 that had been stimulated with rhTNFα or IL1β

Relative expression levels were determined using RT-PCR. All bars indicate the difference compared with the untreated cells (mean expression set at 1). β-actin was used as an endogenous control. Data are shown as the mean ± SEM (n=3). *p<0.05 compared with the rhIL1β-treated HepG2 cells; n.s.: not significant

Adrenic acid as an inflammation enhancer in non-alcoholic fatty liver disease

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Abbreviations used**:**

ACOX (acyl Co-A oxidase), ALT (alanine aminotransferase), AC (acylcarnitine), BMI (body mass index), BSA (bovine serum albumin), CDAHFD (choline deficient L-amino acid defined high fat diet), COL1α1 (collagen type 1 alpha 1), CYP2E1 (cytochrome P450 family 2, subfamily e, polypeptide 1), CYP4A14 (cytochrome P450, family 4, subfamily a, polypeptide 14), ELOVL (elongase), FAS (fatty acid synthase), FATP (fatty acid transport protein), FFA (free fatty acid), GPx1 (glutathione peroxidase-1), IL (interleukin), IL1R (interleukin-1 receptor), LCAD (long chain acyl Co-A dehydrogenase), LC/MS (liquid chromatography/mass spectrometry), LPC (lysophosphatidylcholine), LPE (lysophosphatidylethanolamine), MCAD

(medium chain acyl Co-A dehydrogenase), MCP1 (monocyte chemoattractant protein-1), MIP1β (macrophage inflammatory protein-1 beta), MTTP (microsomal triglyceride transfer protein), NAFLD (non-alcoholic fatty liver disease), NASH (non-alcoholic steatohepatitis), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PUFA (polyunsaturated fatty acid), rh (recombinant human), RT-PCR (real-time reverse transcription polymerase chain reaction), SD (standard diet), TG (triglyceride), TGFβ1 (transforming growth factor-beta 1), TNFα (tumor necrosis factor-alpha), TNFR-1 (TNF receptor-1), UCP2 (uncoupling protein-2), USG (ultrasonography).

Abstract

Background: This study was designed to identify novel links between lipid species and disease progression in non-alcoholic fatty liver disease (NAFLD).

Methods: We analyzed lipid species in the liver and plasma of db/db mice fed a choline-deficient L-amino acid-defined, high-fat diet (CDAHFD) using liquid chromatography/mass spectrometry (LC/MS). An in vitro experiment was performed using HepG2 cells stimulated with recombinant human TNFα or IL1β. The expression of steatosis-, inflammation-, and fibrosis-related genes were analyzed. Plasma samples from NAFLD patients were also analyzed by LC/MS.

Results: The CDAHFD-fed db/db mice with hepatic steatosis, inflammation, mild fibrosis, obesity, and hypercholesterolemia displayed significantly higher hepatic and plasma levels of free adrenic acid ($p<0.05$). The accumulated adrenic acid in the CDAHFD-fed db/db mice was associated with increased expression of ELOVL2 and 5, and the suppression of the acyl-CoA oxidase 1 gene during peroxisomal β-oxidation. The pretreatment of HepG2 cells with adrenic acid enhanced their cytokine-induced cytokines and chemokines mRNA expression. In NAFLD patients, the group with the highest ALT levels exhibited higher plasma adrenic acid concentrations than the other ALT groups (p-value for trend: <0.001).

Conclusion: Data obtained demonstrated that adrenic acid accumulation contributes to disease progression in NAFLD.

Keywords

Liquid chromatography/mass-spectrometry; non-alcoholic fatty liver disease; adrenic acid; chemokine; alanine aminotransferase.

1. Introduction

Epidemiological studies have indicated that the prevalence of non-alcoholic fatty liver disease (NAFLD) is rising in both Western countries and other regions of the world (1,2). Along with the increasing incidence of obesity and its related metabolic diseases, it is predicted that NAFLD will become the major cause of chronic liver disease. Histologically, some NAFLD patients only exhibit steatotic changes in their livers and remain stable for a long period of time, whereas others develop marked liver inflammation and hepatocyte necrosis. The latter subset of patients, which are considered to have non-alcoholic steatohepatitis (NASH), are at increased risk of liver cirrhosis, hepatocellular carcinoma, and liver-related mortality (2–4). Therefore, the early identification of NASH is very important for preventing liver complications.

Disease progression in NAFLD is a complex process involving several factors, including lipid metabolism. Some theories have been postulated to explain how lipids can influence disease progression in NAFLD. The two-hit concept suggests that lipid accumulation serves as a 'first hit' that sensitizes the liver to various other hits, such as oxidative stress, pro-inflammatory cytokines, endotoxins, or hypoxia (5). For example, free cholesterol-sensitized hepatocytes undergo tumor necrosis factor (TNF)-induced apoptosis (6). On the other hand, the lipotoxicity concept suggests that a direct relationship exists between certain lipid species and liver damage. For example, free fatty acids (FFA), such as palmitic acid and lysophosphatidylcholines (LPC), can activate the intrinsic apoptotic pathway through endoplasmic reticulum stress and c-Jun NH2-terminal kinase activation (7,8). According to the multiple-hit concept, this lipotoxicity process together with genetic and dietary factors, adipose tissue dysfunction, and gut flora determine the extent of disease progression in NAFLD (9). On the other hand, ceramides have been also implicated in disease progression, as they promotes insulin resistance, which is a risk factor for NAFLD (10).

Despite extensive investigation of the molecular mechanisms responsible for lipid-related liver damage in NAFLD, the available quantitative data about the use of lipid profiles for differentiating steatohepatitis from simple steatosis are still inconclusive. Metabolomic-based lipid profiling (lipidomic) studies have shown that the fatty acid content of the liver was increased in NAFLD patients compared with controls, but no significant differences were detected between simple steatosis and steatohepatitis (11,12). Similar findings have also been obtained for free cholesterol, total LPC, and ceramide levels (11,13). On the other hand, Gorden et al. reported that the levels of several ceramide species were significantly increased in the plasma, but not the livers, of NASH patients, compared with those seen in simple steatosis patients (14). So far, lipidomic studies have suggested that at least 500 lipid species are present in plasma, and over 1,000 lipid species are found within cells (15,16). Hence, we hypothesized that there might be other lipid species that are pathologically related to NAFLD, and some of them might be useful for differentiating steatohepatitis from simple steatosis. One of the analytical methods used in lipidomic studies is liquid chromatography/mass spectrometry (LC/MS). LC/MS-based analysis makes it possible to profile lipid species accurately from a small minimally pre-treated sample within a short period of time (17). Thus, our aim is to identify novel links between lipid species and the progression of NAFLD using LC/MS.

In this study, we first examined the lipid profiles of genetically obese db/db mice and used a choline deficient-L-amino acid defined-high fat diet (CDAHFD) to induce steatohepatitis in the mice. The CDAHFD induced hepatic steatohepatitis and fibrosis, which are comparable to the pathology of human NASH, in db/db mice. Next, we tested our findings in vitro using HepG2 cells and finally confirmed them using samples from NAFLD patients.

2. Materials and methods

2.1. Ethical approval

All animal treatments in this study were approved by the institutional animal care and use committee and carried out according to the Kobe University animal experimentation regulations. The use of human blood samples was approved by the ethics committees at Kobe University Graduate School of Medicine and its related hospitals, and the analysis of these samples was carried out according to the guidelines of Kobe University Hospital. Informed consent was obtained from all human subjects.

2.2. Animal experiment

Six-week-old male db/db mice were randomly divided into two groups of five mice, which received different dietary formulas for 4 weeks. One group was fed a standard diet (SD; CE-2, CLEA Japan, Inc., Shizuoka, Japan) containing 3,449 kcal/kg. The other group was fed an L-amino-acid-defined, high-fat diet that contained 0.1% methionine but did not contain any choline (CDAHFD; #A06071313). The CDAHFD was purchased from Research Diet, Inc., (New Brunswick, NJ, USA) and contained 5,200 kcal/kg. We also used age-matched male C57BL/6J (B6) mice that were fed the SD for 4 weeks as a control group. The composition of each diet is shown in Supplementary Table S1. The body weights of the mice were recorded every week during the experimental period. On the final day of the experiment, the mice were sacrificed after overnight fasting, and their whole blood was collected by cardiac puncture using heparin as an anticoagulant. The heparinized blood was then centrifuged at 6,000 g for 10 min at 4^{o}C to obtain plasma samples. Before the liver was removed, it was perfused with distilled water and then weighed. A portion of the liver was fixed in 10% formalin buffer for histological evaluation. The plasma samples and remaining portion of the liver were kept at -80^{o}C until further use.

2.3. Liver histology

The formalin-fixed liver tissues were embedded in paraffin, sliced into thin sections, and then stained with standard hematoxylin and eosin (H&E). Hepatic fibrosis was assessed using the picrosirius red stain kit (Polysciences, Inc., USA).

2.4. Measurement of hepatic triglyceride levels

The lipids in the liver tissues of the mice were extracted according to a modified version of the Bligh and Dyer method. Briefly, the liver tissue was homogenized with 9 volumes of 1.15% (w/v) KCl, and aliquots (0.5 mL) of the homogenate were extracted with 2 mL of chloroform-methanol. To remove water-soluble substances, a 1/5 volume of 0.5% (w/v) NaCl was added. After being centrifuged at 1,500 g for 5 min, the resultant chloroform layer was evaporated and then dissolved in methanol supplemented with 10% Triton-X. The hepatic triglyceride (TG) level was quantified using a commercial kit (Wako Pure Chemical Industries, Tokyo, Japan) according to the manufacturer's instructions.

2.5. Measurement of plasma biochemistry

The plasma alanine transferase (ALT), TG, and total cholesterol levels of the mice were measured using commercial kits (Wako Pure Chemical Industries, Tokyo, Japan).

2.6. Fatty acid preparation

The fatty acid solution for the in vitro experiment was prepared by complexing 0.5 mM adrenic acid (Sigma Aldrich, USA) with 1.65% (*w/v*) bovine serum albumin (BSA; Wako Pure Chemical Industries, Tokyo, Japan) in serum-free medium, incubated at 37^{o}C for 1 hr, and then

subjected to filter sterilization. The fatty acid solution was freshly prepared for each experiment. The final molar ratio of the adrenic acid/BSA complex was 2:1.

2.7. **Cell culture conditions and treatment**

HepG2 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako Pure Chemical Industries, Tokyo, Japan) supplemented with 10% (*v/v*) fetal bovine serum (Invitrogen). For the gene expression analysis, HepG2 cells (10^6 cells/well) were seeded onto 6-well plates (Falcon, Corning Inc., USA) and then allowed to rest for 24 hrs. Before the treatment assay, the cells were washed, and the medium was replaced with serum-free medium containing adrenic acid/BSA complex. Control cell cultures were incubated with serum-free medium containing BSA alone. After overnight incubation, the cells were treated with 10 ng/mL recombinant human (rh)TNFα (Reliatech GmbH, Germany) or 5 ng/mL interleukin (IL)1β (PeproTech, USA) in serum-free medium for a further 5 hrs. All cells were incubated in a humidified atmosphere at 37°C/5% CO_2.

2.8. **Quantitative real-time PCR**

Total RNA was extracted from the cultured cells and mouse liver tissue with TRIzol reagent (Invitrogen, Tokyo, Japan) and quantified using an ultraviolet spectrophotometer (ND-1000, NanoDrop, USA). Following the extraction procedure, 1 μg of RNA was treated with the RT^2 first strand kit (Qiaqen, USA) to eliminate contaminating genomic DNA and promote cDNA synthesis. Quantitative real-time PCR was then performed using the 7500 real-time polymerase chain reaction (PCR) system and power SYBR green reagent (Applied Biosystems). The sequences of the primers used for the PCR are listed in Supplementary Table S2. The PCR cycling protocol was as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 sec,

and 60°C for 1 min. Melting curve analysis was conducted after the amplification step to identify a specific PCR product. All mRNA expression levels were normalized to the mRNA expression level of mouse GAPDH or human β-actin. The relative expression levels of all genes were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.9. **Human samples**

After overnight fasting, venous blood samples were collected from 30 patients with NAFLD who were diagnosed during routine health checkups at Hotel Okura Clinic, Kobe, Japan, between April 2016 and September 2016. Blood samples from 10 healthy subjects (i.e., those with a body mass index (BMI) of <23 kg/m^2, normal liver function, and normal metabolic parameters) with normal liver ultrasonographic (USG) findings were also collected. All subjects underwent clinical, hematological, biochemical, and serological evaluations during the health checkup. For the fatty acid analysis, plasma was prepared and kept at -80°C. The diagnosis of NAFLD was based on USG findings : increased hepato-renal echo contrast with reduced penetration of the posterior segment of the right lobe and poor visualization of the hepatic vessels and diaphragm. A normal liver was defined as a liver with a homogenous parenchyma that exhibited similar or slightly higher echogenicity than the renal cortex and well-visualized hepatic vessels and diaphragm. The USG examinations were performed by experienced sonographers using a Siemens ACUSON S1000 (Siemens, USA), and the resultant images were reviewed independently by certified gastroenterologists. None of the patients had a history of alcoholism (>20 g alcohol/day), steatogenic drug use, viral hepatitis, autoimmunity, malignancy, hepatobiliary disease, or other chronic liver diseases.

The patients were divided into three groups based on their ALT levels: group 1: ALT <30 IU/L for males, ALT <20 IU/L for females; group 2: 30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females; group 3: ALT ≥60 IU/L for males, ≥40 IU/L for females.

2.10. **Liquid chromatography-mass spectrometry (LC/MS)-based lipid analysis**

The lipid species present in the mouse and human samples were analyzed by LC/MS. Frozen mouse liver tissue samples (~5 mg) were homogenized in 225 μL of methanol and 25 μL di-lauroyl phosphatidylcholine (the internal standard). Then, 80 μL of methanol and 10 μL of internal standard were added to the plasma samples (10 μL) and vortexed. Next, the samples were kept on ice for 10 min. After being centrifuged (16,000 g, 4°C, 5 min), the supernatants (25 μL) were collected and transferred to vials for analysis. The LC/MS analysis was performed on a Nexera LC system coupled to an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corp., Japan). The lipid species were separated using an octadecyl silylated silica column (InertSustain C18, 100 x 2.1 mm, 3 μm, GL Sciences, Tokyo, Japan) with a guard column (10 x 3 mm, 5 μm). The peak areas under the curve of each lipid species were detected and then normalized to the internal standard. For the lipids extracted from liver tissue, after the data had been normalized, the relative amount was then calculated based on the weight of liver tissue prepared during the extraction process.

2.11. **Statistical analysis**

The significance of differences was analyzed using the Student's t-test or one-way ANOVA (SPSS version 22; SPSS, Inc., Chicago, IL, USA). For the human lipid data, comparisons of group means were conducted with one-way ANOVA and the Tukey honest significant difference test for across-group comparisons. A linear contrast analysis for ANOVA was also performed to

test for linear trends in the mean values. P-values or p-values for trends of <0.05 were regarded as statistically significant. Unless stated otherwise, all data are presented as the mean ± standard error of mean (SEM).

3. Results

3.1. Steatohepatitis and fibrosis were induced in the db/db mice fed the CDAHFD

After 4 weeks' feeding, benign hepatic steatosis without inflammation was observed in the livers of the SD-fed db/db mice. On the other hand, severe steatosis, inflammatory foci, as well as hepatocyte ballooning, were seen in the livers of the CDAHFD-fed db/db mice (Figure 1). In agreement with this, the CDAHFD-fed db/db mice exhibited significantly higher hepatic mRNA levels of TNFα and CD68. Sirius red staining performed after 4 weeks' feeding showed mild perisinusoidal fibrosis, which was indicative of an early stage of hepatic fibrosis, in the CDAHFD-fed db/db mice, but not the SD fed mice. In addition, the hepatic expression levels of transforming growth factor (TGF)β1 and collagen (COL)1α1 were significantly increased in the CDAHFD-fed db/db mice. The metabolic abnormalities seen in each group of mice are summarized in Table 1. Significant increases in body weight, liver weight, the hepatic TG level, the plasma total cholesterol level, and the ALT level were observed in the CDAHFD-fed db/db mice compared with the SD-fed db/db mice, while the plasma TG level did not differ significantly between the two groups (P=0.61).

3.2. Oxidative stress-related gene expression

To evaluate hepatic oxidative stress, we measured the hepatic mRNA levels of uncoupling protein-2 (UCP2) and glutathione peroxidase-1 (GPx1). Compared with the SD-fed db/db mice, the consumption of the CDAHFD diet was associated with the downregulation of hepatic GPx1

mRNA expression and the upregulation of hepatic UCP2 mRNA expression. The baseline hepatic GPx1 mRNA level of the SD-fed db/db mice was about three times higher than that of the B6 control mice, whereas their baseline hepatic UCP2 mRNA level was significantly lower than that seen in the B6 control mice (Table 1).

3.3. **Lipid metabolism-related gene expression**

The baseline hepatic fatty acid synthase (FAS) mRNA level of the db/db mice fed the SD was lower than that seen in the B6 control mice (Figure 2). Although the difference was not statistically significant, the CDAHFD-fed db/db mice displayed an even lower hepatic FAS mRNA level (P=0.061). The hepatic mRNA levels of medium- (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD), which are enzymes that are involved in the initial stages of mitochondrial β-oxidation, did not differ significantly between the two db/db mouse groups. On the other hand, the CDAHFD-fed db/db mice exhibited lower hepatic mRNA levels of acyl-CoA oxidase (ACOX1), which is an enzyme that is involved in the initial stages of peroxisomal β-oxidation, than the SD-fed db/db mice. The SD-fed db/db mice displayed a significantly lower baseline hepatic mRNA level of microsomal TG transfer protein (MTTP) than the B6 control mice. Feeding the db/db mice the CDAHFD did not increase their MTTP mRNA expression. Compared with those seen in the B6 control mice, the baseline hepatic mRNA levels of cytochrome P450 2E1 (CYP2E1) and cytochrome P450, family 4, subfamily a, polypeptide 14 (CYP4A14) of the SD-fed db/db mice were significantly higher and lower, respectively. Feeding the db/db mice the CDAHFD did not increase the mRNA expression level of either enzyme. In the SD-fed db/db mice, the baseline hepatic mRNA level of fatty acid transport protein 2 (FATP2) was significantly lower than that seen in the B6 control mice, while the baseline hepatic

mRNA level of FATP5 was increased. The hepatic mRNA levels of both FATP2 and 5 were significantly decreased in the CDAHFD-fed db/db mice.

3.4. **Lipid profiles of the liver and plasma**

In total, 236 species from 6 classes of lipids (phosphatidylcholines, PC; phosphatidylethanolamines, PE; LPC; lysophosphatidylethanolamines, LPE; FFA; acylcarnitines, AC) were detected in the liver and plasma samples (Supplementary Tables S3 & S4). Compared with those seen in the B6 control mice, the hepatic levels of 115 lipid species exhibited significant changes in the SD-fed db/db mice; i.e., the levels of 90 species were downregulated, and those of 25 were elevated. In a comparison between the CDAHFD-fed and SD-fed db/db mice, significant differences in the hepatic levels of 116 lipid species were detected; i.e., the hepatic levels of 73 and 43 species were downregulated and upregulated, respectively, in the CDAHFD-fed mice. Of the hepatic lipid species that exhibited fold-change values of greater than or less than 2 between the SD-fed db/db and B6 control mice or between the CDAHFD-fed and SD-fed db/db mice, the 6 in each fold-change category whose intergroup differences were most significant are shown in Table 2.

In the analysis of the plasma samples, 130 out of 236 lipid species demonstrated significant differences in their plasma levels between the SD-fed db/db mice and the B6 control mice, and 98 of these species were downregulated in the SD-fed db/db mice. When we compared the plasma samples of the SD-fed db/db mice with those of the CDAHFD-fed db/db mice, we found that 139 lipid species exhibited significantly different levels between the two groups, with 43 lipid species demonstrating lower levels in the CDAHFD-fed db/db mice. Of the plasma lipid species that exhibited fold-change values of greater than or less than 2 between the SD-fed db/db

and B6 control mice or between the CDAHFD-fed and SD-fed db/db mice, the 6 in each fold-change category whose intergroup differences were most significant are shown in Table 3.

In a detailed examination of the lipid profiles of the mice, we found that the plasma level of free eicosapentaenoic acid (20:5n3) was higher in the SD-fed db/db mice than in the B6 control mice. Similarly, we also found that the plasma and hepatic levels of phospholipids and lysophospholipids containing 20:5n3 were also increased in the SD-fed db/db mice. Interestingly, the hepatic level of free adrenic acid (22:4n6) was markedly higher in the CDAHFD-fed db/db mice than in the SD-fed db/db mice. In plasma, the difference in the adrenic acid level reached statistical significance ($p<0.001$). We also found that the concentrations of phospholipid species containing adrenic acid, such as PC or PE (40:4) (containing side chain 18:0/22:4), and LPC 22:4 (sn-1/sn-2), were also significantly increased. The hepatic and plasma levels of adrenic acid, which is an omega 6 polyunsaturated fatty acid (PUFA), exhibited greater differences between the CDAHFD-fed db/db mice and the SD-fed db/db mice (8.6-fold higher vs. 1.6-fold higher in liver tissue, 5.3-fold higher vs. 2.2-fold higher in plasma) than those of well-known pro-inflammatory PUFA, such as arachidonic acid (Figure 3). Since the contribution of adrenic acid to NAFLD has not been examined in detail, we next focused on elucidating its potential role in the progression of the disease.

3.5. **Increased mRNA expression of elongase 2 and 5 in the CDAHFD-fed db/db mice**

First, in order to understand why the hepatic level of adrenic acid was markedly increased in the CDAHFD-fed db/db mice, the hepatic mRNA levels of ELOVL2 and 5 were examined using quantitative real-time PCR (Figure 4). Adrenic acid is produced via the elongation of arachidonic acid by ELOVL2 and 5. We detected significantly higher hepatic mRNA levels of ELOVL2 and 5 in the CDAHFD-fed db/db mice than in the SD-fed db/db mice. However, the SD-fed db/db

mice displayed significantly lower basal hepatic expression levels of ELOVL2 and 5 than the B6 control mice.

3.6. Enhancement of proinflammatory cytokine-induced mRNA expression in adrenic acid-pretreated HepG2 cells

To confirm the role of adrenic acid in inflammation, we first pretreated HepG2 cells with 0.5 mM of adrenic acid before stimulating them with rhTNFα or IL1β (Figures 5 & 6). Compared with TNFα treatment alone, the HepG2 cells that were pretreated with adrenic acid and then treated with TNFα expressed higher mRNA levels of TNFα, IL8, macrophage inflammatory protein 1β (MIP1β), and monocyte chemoattractant protein 1 (MCP1). Under IL1β stimulation, the HepG2 cells that were pretreated with adrenic acid and then treated with IL1β expressed higher mRNA levels of TNFα, IL8, MIP1β, and TGFβ1, but lower MCP1 mRNA levels.

3.7. Changes in the expression of IL-1 receptors in adrenic acid-pretreated HepG2 cells

We next examined whether adrenic acid induced cytokine receptor expression (Figure 7). The mRNA level of TNF receptor type I (TNFR-1) was not significantly upregulated in adrenic acid-pretreated HepG2 cells that were subsequently treated with rhTNFα. In contrast, the mRNA expression of the type I IL-1 receptor fell significantly, and that of the type II IL-1 receptor increased significantly after the cells were treated with rhTNFα.

3.8. Association between the plasma levels of adrenic acid and ALT in human NAFLD

Finally, we focused on measuring the relative abundance of FFA in the plasma of NAFLD patients and comparing these values among three groups based on the NAFLD patients' ALT levels. Overall, the NAFLD patients were older, had higher BMI, and were more dyslipidemic than the normal liver patients. There were no significant differences in age, gender, BMI, lipid or

glucose parameters, or liver function among the NAFLD patients (Table 4). ANOVA showed that the mean plasma levels of palmitic acid (16:0), stearic acid (18:0), arachidic acid (20:0), erucic acid (22:1n9), eicosadienoic acid (20:2n6), adrenic acid (22:4n6), docosapentaenoic acid (22:5n6), stearidonic acid (18:4n3), eicosapentaenoic acid (20:5n3), and docosahexaenoic acid (22:6n3) differed significantly between the groups (Table 5). All of these fatty acids, except for 20:0 and 22:1n9, exhibited significant positive trends across the groups. Erucic acid (22:1n9) displayed a decreasing trend as the ALT increased, but it did not reach the prespecified 0.05 significance level for linear trends (p=0.057). Post-hoc analysis showed that only adrenic acid (22:4n6) and docosapentaenoic acid (22:5n6) displayed significantly higher mean values in group 3 (ALT ≥60 IU/L for males; ALT ≥40 IU/L for females) than in the normal liver group or the group 1 (ALT <30 IU/L for males, <20 IU/L for females) or group 2 (30 ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females) NAFLD patients.

4. **Discussion**

The main findings of this study are as follows: (i) the plasma and hepatic levels of free adrenic acid were markedly increased in a mouse model of steatohepatitis, but not in a mouse model of simple steatosis; (ii) the increased hepatic levels of adrenic acid were due to increased endogenous synthesis and decreased catabolism in peroxisomes; (iii) pretreatment with adrenic acid was associated with enhanced mRNA expression of proinflammatory molecules in cytokine-stimulated hepatocytes; (iv) high ALT levels (≥60 IU/L in males, ≥ 40 IU/L in females) were associated with higher plasma adrenic acid levels in NAFLD patients.

A previous study showed that the CDAHFD induced fibrotic steatohepatitis in the livers of C57BL/6J mice (18). In our study, the CDAHFD also induced steatohepatitis and fibrosis in db/db mice, as confirmed histologically and through gene expression analysis. However, in

contrast with the methionine choline-deficient diet, the CDAHFD induced body weight gains in the db/db mice. In addition, the total cholesterol level of the CDAHFD-fed mice also rose. Therefore, this group of mice exhibits a NASH-like phenotype combined with obesity and hypercholesterolemia, two features which are commonly found in human NAFLD. NASH is also associated with oxidative stress due to an imbalance between the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and antioxidant activity, such as that of GPx. Oxidative stress can cause impaired mitochondrial oxidative phosphorylation, leading to the upregulation of the expression of UCP2, an uncoupling protein (19,20). In our study, we did not directly measure H_2O_2 production, but the increased expression of GPx1 seen in the SD-fed db/db mice suggests that hepatic steatosis is associated with increased ROS production. Thus, increased expression of GPx1 would have helped the SD-fed db/db mice to keep their redox state in balance, and therefore, UCP2 mRNA expression was not markedly induced in these mice. On the other hand, the CDAHFD diet suppressed GPx1 mRNA expression, which might have tilted the redox state of the CDAHFD-fed mice toward ROS production. As an adaptive response, UCP2 mRNA expression was upregulated and might have caused proton leakage across the inner mitochondrial membrane and impaired ATP production. Overall, this process could be associated with inflammation, liver damage, and increased plasma ALT levels in CDAHFD-fed db/db mice.

Hepatic steatosis can be caused by the impairment of several metabolic pathways, such as lipid uptake (FATP2 & 5), de novo lipogenesis (FAS), mitochondrial β-oxidation (MCAD, LCAD), peroxisomal β-oxidation (ACOX1), microsomal ω-oxidation (CYP2E1, CYP4A14), and lipid export (MTTP). The results of the present gene expression analysis indicated that the impaired secretion of TG-rich very low density lipoproteins and increased fatty acid uptake in

the liver were associated with hepatic steatosis in our simple steatosis model. On the other hand, additional impairment of peroxisomal β-oxidation without further upregulation of microsomal ω-oxidation contributed to the severe steatosis observed in the livers of our steatohepatitis model. The absence of a further upregulation of CYP2E1 and CYP4A14 mRNA expression in our steatohepatitis model might have been due to inflammation. Previous studies have shown that hepatic CYP2E1 and CYP4A14 expression were suppressed by inflammatory cytokines, such as TNFα (21,22).

Lipid analysis of NAFLD has consistently shown an increase in the n6/n3 ratio of PUFA, mainly due to the depletion of n3 species. However, the exact alterations in the level of each n6 PUFA remain to be elucidated. Yamada et al. showed that the levels of n6 PUFA, such as linoleic acid (18:2n6) and docosadienoic acid (22:2n6), were higher in liver tissue from patients with simple steatosis or steatohepatitis than in control liver tissue (12), while others found that arachidonic acid exhibited relatively low levels within several lipid classes (11,23,24). In the plasma of patients with simple steatosis and steatohepatitis, the concentration of linoleic acid was decreased while those of gamma-linolenic acid (18:3n6) and dihomo-gamma-linolenic acid (20:3n6) were increased (25). In our study, the CDAHFD-fed db/db mice demonstrated significantly higher levels of eicosadienoic acid (20:2n6), arachidonic acid (20:4n6), and adrenic acid (22:4n6) than the SD-fed db/db mice, and adrenic acid (22:4n6) exhibited the biggest differences between the groups. Furthermore, the levels of some phospholipid species containing adrenic acid were also significantly increased. Taken together, these data suggest that n6 PUFA synthesis was increased in the livers of our steatohepatitis model, which resulted in adrenic acid enrichment. Increased plasma total adrenic acid levels, which include both the esterified type and the unesterified free type of adrenic acid, have been detected in NASH patients (26). The plasma

concentrations of phospholipids containing adrenic acid were also increased in NASH-associated hepatocellular carcinoma patients (27). In our study, the plasma level of free adrenic acid was increased in mice with steatohepatitis and NAFLD patients. The abovementioned studies and our results strongly suggest that adrenic acid contributes to steatohepatitis.

Endogenous adrenic acid is produced via the elongation of arachidonic acid by ELOVL2 or 5, and it is predominantly oxidized or chain-shortened in peroxisomes (28,29). ACOX catalyzes the first step in peroxisomal β-oxidation. In mammals, there at least three isoforms of ACOX: ACOX1, ACOX2, and ACOX3 (30), and adrenic acid is a substrate for ACOX1 (31). Compared with those seen in the SD-fed db/db mice, the ACOX1 mRNA level was decreased in the CDAHFD-fed db/db mice, but the expression levels of ELOVL2 and 5 were increased. These data suggest that the higher hepatic and plasma levels of adrenic acid seen in the steatohepatitis model were caused by both increased endogenous synthesis and decreased catabolism in the hepatic peroxisomes.

Peroxisomes are extramitochondrial organelles that participate in fatty acid oxidation in the liver, as does the cytochrome P450-containing endoplasmic reticulum. An early study detected greater peroxisome-based β-oxidation in genetically obese mice than in lean mice (32). However, the impairment of peroxisome β-oxidation might be a key factor in the progression of simple steatosis to steatohepatitis. Mice deficient in ACOX genes were found to develop severe hepatic steatosis from a young age. By 5 months of age, the ACOX-deficient mice displayed hepatocyte apoptosis and lipogranuloma formation, which subsequently progressed to hepatocellular carcinoma (33). Meanwhile, Mitsuyoshi et al. detected lower hepatic expression of ACOX in NASH patients than in patients with simple steatosis and demonstrated that hepatic ACOX

expression decreased further as the disease progressed (34). However, adrenic acid levels were not investigated in these studies.

ELOVL2 and 5 are very long chain fatty acid elongase enzymes. Both are expressed in the liver, where C20 and C22 PUFA are elongated by ELOVL2, while ELOVL5 elongates C18 and C20 PUFA (35). There is a lack of convincing evidence regarding the promotion of NASH by ELOVL2 or 5, but lower hepatic expression of ELOVL5 has been reported in mice with high fat diet-induced fatty liver (36). A study by Moon et al. suggested that the deletion of ELOVL5 increased the nuclear abundance of sterol regulatory element-binding transcription factor 1 and hepatic steatosis by depleting docosahexaenoic acid (DHA, 22:6n3) (37). The deletion of ELOVL2 also causes the DHA depletion and upregulated expression of lipogenic genes, albeit without leading to hepatic steatosis (38). In our study, we also found a trend towards lower DHA levels in the steatotic livers of the SD-fed db/db mice compared with the B6 control mice. Although the CDAHFD-fed db/db mice exhibited higher ELOVL2 and 5 expression, the relative abundance of DHA in the CDAHFD-fed db/db mice did not differ significantly from that seen in the SD-fed db/db mice. This might have been due to impaired peroxisomal β-oxidation, as DHA is generated via the oxidation of tetracosahexaenoic acid (24:6n3) in peroxisomes.

In our study, the db/db mice fed the SD did not instantly develop hepatic steatohepatitis despite suffering from marked obesity compared with the B6 control mice. While this finding is in agreement with a previous study (39), there is still no firm explanation for it. Our lipid analysis suggests that the above fidning could be due to the increased abundance of eicosapentaenoic acid (20:5n3), an antiinflammatory lipid. db/db mice have a functional defect in the long form leptin receptor, which impairs satiety, and hence, leptin can not inhibit food intake (40). Although the SD-fed db/db mice consumed a similar type of diet to the B6 control mice and

exhibited lower basal expression of ELOVL2 and 5, this might have resulted in the saturation of ELOVL2 and 5 reactions, leading to the accumulation of eicosapentaenoic acid (20:5n3).

The presence of inflammation can predict the progression of NASH to advanced fibrosis (41). Therefore, we investigated the influence of adrenic acid on proinflammatory gene expression using HepG2 cells (a hepatocyte model). In this study, the pretreatment of HepG2 cells with adrenic acid augmented autocrine TNFα expression. Elevated TNFα levels have been detected in the blood and livers of NAFLD patients (42,43). We also found that adrenic acid pretreatment enhanced the mRNA expression levels of TNFα-induced chemokines, such as IL8, MIP1β, and MCP1. The main function of chemokines is to attract immune cells to inflammatory sites. Chemokines themselves have been implicated in several inflammatory diseases, including those with metabolic components, such as atherosclerosis, obesity, and diabetes (44). In human NAFLD, Bertola et al. detected increased hepatic mRNA expression of several chemokines, including IL8, MIP1β, and MCP1 (45). Accordingly, the liver pathology of NASH usually involves the infiltration of a mixture of inflammatory cells, such as neutrophils, monocytes, and macrophages (46). Elevated IL1β levels have also been detected in an animal model of NASH and human NASH (47,48). Therefore, we also evaluated gene expression under IL1β treatment. Compared with TNFα, IL1β is a more potent inducer of TNFα, IL8, MIP1β, and MCP1 expression in HepG2 cells. The mRNA levels of all of these chemokines, except for MCP1, were upregulated in HepG2 cells and further enhanced in adrenic acid-pretreated HepG2 cells. This suggests that the upregulating effect of adrenic acid on MCP1 mRNA expression during inflammation is cytokine-specific.

The inflammatory activities of TNFα and IL1β are mediated by type 1 TNF receptors (TNFR-1) and type 1 IL-1 receptors (IL1R-1), respectively. On the other hand, type 2 IL-1

receptors (IL1R-2) act as “decoy receptors” and inhibit IL1β activity (49). Gene expression analysis of these receptors in adrenic acid-pretreated HepG2 cells indicated that: (i) negative regulation by IL1R-2 inhibits IL1β-induced inflammation, and (ii) adrenic acid might modify TNFα or IL1β signaling pathways at the post-receptor level. The induction of chemokine expression by TNFα or IL1β is dependent on the activation of NF-kappaB and activator protein-1 (AP-1). It is possible that the release of free adrenic acid (or related metabolites) after TNFα or IL1β stimulation leads to sustained activation of NF-kappaB or AP-1. Therefore, further studies are needed to investigate the effects of free adrenic acid on NF-kappaB and the AP-1 signaling pathway.

In our in vitro study, neither TNFα nor IL1β induced TGFβ1 mRNA expression in HepG2 cells. However, it was significantly increased in adrenic acid-pretreated HepG2 cells after IL1β treatment. It is possible that the more intense inflammation seen in adrenic acid-pretreated HepG2 cells after IL1β treatment is accompanied by hepatocyte damage and increased oxidative stress, which subsequently stimulates TGFβ1 mRNA expression. Hepatocyte damage-induced TGFβ1 release could enhance stellate cell activation, leading to extracellular matrix synthesis and hepatic fibrosis (50,51). Besides TGFβ1, several pieces of evidence also suggest that chemokines themselves can act as profibrogenic mediators. Liver stellate cells express C-C chemokine receptor type 2 (CCR2) (52) and CCR5 (53), which are receptors for MCP1 and MIP1β, respectively. Stellate cells can be activated by IL8 (54), while MCP1 and MIP1β can induce their migration (52,55,56). These findings suggest that adrenic acid might also influence the progression of fibrosis by upregulating the expression of profibrotic chemokines in hepatocytes.

To confirm the relationship between increased adrenic acid production and liver inflammation in the clinical setting, we examined the association between plasma adrenic acid levels and ALT levels in NAFLD patients. A previous study by Suzuki et al. showed that changes in the serum ALT levels of NASH patients were correlated with liver inflammation, in both univariate and multivariate analyses (57). Therefore, we divided our patients into three groups based on their ALT levels (different cut-off values were employed for males and females). In our data analysis, we found that the mean plasma adrenic acid level was higher in group 3 (ALT ≥60 IU/L for males, ≥40 IU/L for females) than in group 1 (ALT <30 IU/L for males, <20 IU/L for females) and group 2 (30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females). These intergroup differences and the associated linear trend were statistically significant. Overall, these findings support our in vitro results. We also obtained a similar finding with regard to the plasma level of docosapentaenoic acid (22:5n6) in our human data set. Since the formation of docosapentenoic acid (22:5n6) involves β-oxidation in peroxisomes, this might indicate that our patients had mild disease that did not involve significant changes in peroxisome β-oxidation, as shown by the mouse model. A positive correlation between serum FFA levels and ALT was detected in NAFLD in a previous study (58). Our results provide more information by showing which fatty acids are associated with increased ALT levels in NAFLD. The limitations of our human sample analysis include the small number of patients, the lack of histological examinations, and the fact that it only included patients from one medical center.

In summary, the combination of increased PUFA and elongase expression and impaired peroxisomal β-oxidation cause the accumulation of adrenic acid in experimental steatohepatitis, but not in simple steatosis. Under inflammatory conditions, adrenic acid might exacerbate inflammation by enhancing the expression of chemokine genes in hepatocytes. Increased plasma

adrenic acid levels are associated with high ALT levels in NAFLD patients. Taken together, adrenic acid accumulation contributes to disease progression in NAFLD. A further study is needed to determine whether adrenic acid measurements could be used as a diagnostic biomarker of steatohepatitis in NAFLD.

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Disclosure Statement

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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Table 1. Metabolic features and hepatic mRNA expression levels of db/db mice fed the SD or CDAHFD at 4 weeks

	C57BL/6J	db/db	
Diet	SD	SD	CDAHFD
Body weight (g)			
Initial	21.0 ± 0.3	37.4 ± 0.5*	36.6 ± 1.1
Final	25.6 ± 0.2	42.5 ± 1.3*	51.0 ± 2.6#
Liver weight (g)	2.0 ± 0.06	3.5 ± 0.1*	5.05 ± 0.7#
Hepatic TG (mg/g)	12.7 ± 0.7	26.4 ± 2.7*	69.3 ± 9.3#
Plasma TG (mg/dl)	27.5 ± 4.8	120.4 ± 14.7*	111.3 ± 8.5
Plasma total cholesterol (mg/dl)	89.2 ± 1.7	128.1 ± 6.3*	227.4 ± 9.6#
Plasma ALT (IU/L)	12.3 ± 1.4	18.9 ± 4.3	110.7 ± 8.6#
Hepatic mRNA expression			
TNFα	1.0 ± 0.3	0.8 ± 0.2	2.9 ± 0.5#
CD68	1.0 ± 0.1	0.3 ± 0.03*	1.7 ± 0.3#
TGFβ1	1.0 ± 0.02	1.1 ± 0.2	2.0 ± 0.1#
COL1α1	1.0 ± 0.2	0.5 ± 0.1*	4.2 ± 0.6#
GPx1	1.0 ± 0.03	2.9 ± 0.3*	1.0 ± 0.15#
UCP2	1.0 ± 0.1	0.5 ± 0.08*	1.1 ± 0.17#

SD, standard diet; CDAHFD, choline deficient L-amino acid defined high fat diet; TG, triglyceride; ALT, alanine aminotransferase; TNFα, tumor necrosis factor-alpha; TGFβ1, transforming growth factor-beta 1; COL1α1, collagen type 1, alpha 1; GPx1, glutathione peroxidase-1; UCP2, uncoupling protein-2;

Hepatic mRNA levels were analyzed by RT-PCR. GAPDH was used as an endogenous control. Data are shown as mean ± SEM (n=5) values. *$p<0.05$ compared with the C57BL/6J, SD mice; # $p<0.05$ compared with the db/db, SD mice

Table 2. The six lipid species in the <2 and >2 fold-change categories that exhibited the most significant intergroup differences in their hepatic levels

Species	Fold (SS/CT)	P-value	Species	Fold (SH/SS)	P-value
PE (16:1/20:5)	4.52	0.0051	FA 22:4n6	8.67	0.0045
LPC 20:5 (sn-1)	4.23	0.0274	LPC 22:4 (sn-1)	5.72	0.0026
LPE 20:5 (sn-1)	3.49	0.0076	PC (20:1/20:3_18:0/22:4)	4.99	<0.001
LPC 20:3 (sn-1)	3.41	0.0025	PE (16:0p/20:4)	3.5	<0.001
PC (18:1/20:2_18:0/20:3)	3.05	0.0028	PC (18:1e/20:3)	2.85	0.0092
PE (16:0/20:5)	3.03	<0.001	PE (18:0p/20:4)	2.75	<0.001
PE (20:0e/18:1)	0.13	0.0205	PE (16:1/20:5)	0.06	0.0025
PE (18:0/22:4_20:0/20:4)	0.15	0.0137	LPE 20:5 (sn-1)	0.1	0.0037
PE (19:0/20:4)	0.17	<0.001	PC (15:0/20:5)	0.13	0.0070
PE (20:1/22:6)	0.22	0.0014	LPC 20:5 (sn-1)	0.16	0.0203
PC (18:1e/22:5)	0.26	0.0832	PE (18:0/20:5)	0.19	<0.001
PC (18:0p/18:1_18:1e/18:1)	0.26	0.0017	PC (16:1/20:5)	0.19	0.0140

CT, controls (C57BL/6J mice fed the SD); SS, simple steatosis (db/db mice fed the SD); SH, steatohepatitis (db/db mice fed the CDAHFD); PE, phosphatidylethanolamines; PC, phosphatidylcholines; LPC; lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; FA, fatty acids

P-values were obtained using the Student's t-test.

Table 3. The six lipid species in the <2 and >2 fold-change categories that exhibited the most significant intergroup differences in their plasma levels

Species	Fold (SS/CT)	P-value	Species	Fold (SH/SS)	P-value
PC (16:0/22:2)	6.52	<0.001	PC (20:1/20:3_18:0/22:4)	10.99	<0.001
LPE 20:5 (sn-2)	6.37	0.0014	LPC 22:4 (sn-1)	9.36	<0.001
LPE 20:5 (sn-1)	3.92	0.0199	LPC 22:4 (sn-2)	7.94	<0.001
FA 20:5n3	3.15	<0.001	PE (18:0/22:4_20:0/20:4)	7.32	0.0077
LPC 20:5 (sn-2)	2.8	0.0231	PC (18:1e/20:5)	6.62	0.0117
PC (18:1/20:3)	2.7	<0.001	FA 22:4n6	5.33	<0.001
PC (18:1e/20:5)	0.12	<0.001	LPE 20:5 (sn-1)	0.18	0.0152
PE (20:0e/20:4_18:0e/22:4)	0.23	0.0243	PE (16:0p/20:5)	0.18	0.0740
PE (17:0/22:6)	0.23	<0.001	LPC 20:5 (sn-2)	0.23	0.0128
PE (16:0p/20:4)	0.26	<0.001	PE (16:1/20:5)	0.24	0.0157
PE (17:0/20:4)	0.27	0.0012	PE (18:1p/20:3_16:0p/22:4)	0.24	<0.001
PE (16:0/22:4)	0.28	0.0274	PE (18:0/20:5)	0.25	<0.001

CT, control (C57BL/6J mice fed the SD); SS, simple steatosis (db/db mice fed the SD; SH, steatohepatitis (db/db mice fed the CDAHFD). PE, phosphatidylethanolamines; PC, phosphatidylcholines; LPC; lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; FA, fatty acids

P-values were obtained using the Student's t-test

Table 4. Characteristics of USG-diagnosed NAFLD patients according to their ALT levels

	Normal liver	NAFLD		
		Group 1	Group 2	Group 3
	(n=10)	(n=10)	(n=10)	(n=10)
Sex (F/M)	5/5	6/4	5/5	6/4
Age (years)	39.5 ± 1.3	51.7 ± 2.2*	47.2 ± 1.9*	47.8 ± 1.8*
BMI (kg/m^2)	19.6 ± 0.5	29.1 ± 0.8*	27.0 ± 0.5*	28.4 ± 1.2*
AST (IU/L)	19.0 ± 1.3	18.1 ± 1.3	23.9 ± 0.9	50.3 ± 6.6*#
ALT (IU/L)	17.2 ± 2.0	17.8 ± 1.6	35.2 ± 1.4	77.1 ± 13.5*#
Albumin (g/dl)	4.5 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
Total bilirubin (mg/dl)	0.8 ± 0.1	0.8 ± 0.05	0.8 ± 0.07	0.7 ± 0.06
Platelets (x10^4/mm^3)	22.2 ± 0.8	25 ± 1.3	27.4 ± 2.8	26.2 ± 1.6
TG (mg/dl)	74.1 ± 12.4	122.7 ± 15.3	155.6 ± 25	199.4 ± 29.3*
Total cholesterol (mg/dl)	181.5 ± 6.3	213.8 ± 10.3	221.5 ± 9.2*	230.5 ± 12.1*
LDL (mg/dl)	102.1 ± 6.2	138.1 ± 8.7*	142.6 ± 6.4*	146.4 ± 10.6*
HDL (mg/dl)	72.1 ± 4.2	59.8 ± 4.9	58 ± 3.9	56.1 ± 3.1*
Fasting glucose (mg/dl)	93.5 ± 3.4	98.4 ± 2.3	100 ± 2.6	99.5 ± 2.0
HbA1c (%)	5.4 ± 0.1	5.7 ± 0.06*	5.6 ± 0.07	5.8 ± 0.1*

Data are shown as the mean ± SEM. Group 1: ALT <30 IU/L for males, ALT <20 IU/L for females; group 2: 30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females; group 3: ALT ≥60 IU/L for males, ≥40 IU/L for females

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine transaminase; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin

* $p<0.05$ compared with the normal liver group; # $p<0.05$ compared with groups 1 and 2

Table 5. Relative abundance of plasma FFA in NAFLD patients based on their ALT levels

	Normal liver		NAFLD						P-value	P-value for trend
			Group 1		Group 2		Group 3			
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM		
Saturated fatty acids										
12:0	0.077	0.027	0.086	0.008	0.07	0.006	0.074	0.006	0.488	0.452
14:0	0.372	0.052	0.388	0.038	0.386	0.041	0.49	0.062	0.319	0.121
16:0	13.407^{b}	1.342	16.706^{ab}	1.17	16.161^{ab}	1.349	20.084^{a}	1.671	**0.017**	**0.004**
17:0	0.078	0.011	0.071	0.006	0.083	0.01	0.082	0.007	0.768	0.605
18:0	7.731^{b}	0.691	8.617^{ab}	0.391	8.175^{b}	0.665	10.542^{a}	0.591	**0.011**	**0.005**
20:0	0.075^{a}	0.009	0.062^{ab}	0.003	0.049^{b}	0.002	0.062^{ab}	0.004	**0.013**	**0.033**
21:0	0.023	0.003	0.019	0.002	0.015	0.002	0.024	0.004	0.074	0.777
22:0	0.114	0.015	0.09	0.006	0.087	0.005	0.084	0.007	0.104	**0.032**
23:0	0.048	0.007	0.042	0.005	0.037	0.004	0.046	0.006	0.525	0.622
Monounsaturated fatty acids										
14:1n5	0.124	0.024	0.114	0.013	0.118	0.009	0.15	0.012	0.41	0.279
16:1n7	1.512	0.328	1.645	0.24	1.518	0.139	2.126	0.278	0.295	0.144
17:1n7	0.235	0.036	0.241	0.024	0.259	0.019	0.307	0.031	0.283	0.075
20:1n9	0.619	0.093	0.606	0.095	0.569	0.054	0.701	0.086	0.721	0.581
22:1n9	0.200^{a}	0.029	0.151^{ab}	0.026	0.075^{b}	0.018	0.142^{ab}	0.038	**0.031**	0.057
24:1n9	0.166	0.019	0.180	0.024	0.145	0.01	0.163	0.017	0.598	0.585
Polyunsaturated fatty acids										

18:2n6	6.974	0.927	7.747	0.786	7.422	0.645	8.59	0.717	0.516	0.201
20:2n6	0.226[b]	0.03	0.249[ab]	0.023	0.274[ab]	0.021	0.342[a]	0.036	**0.039**	**0.006**
20:4n6	0.466	0.107	0.524	0.037	0.513	0.042	0.664	0.05	0.182	0.054
22:4n6	0.065[b]	0.005	0.104[b]	0.009	0.103[b]	0.015	0.147[a]	0.01	**<0.001**	**<0.001**
22:5n6	0.215[b]	0.019	0.306[b]	0.024	0.326[b]	0.035	0.479[a]	0.046	**<0.001**	**<0.001**
18:4n3	0.016[b]	0.003	0.027[ab]	0.005	0.018[b]	0.002	0.039[a]	0.007	**0.006**	**0.009**
20:5n3	0.048[b]	0.005	0.076[ab]	0.012	0.075[ab]	0.011	0.113[a]	0.02	**0.013**	**0.002**
22:6n3	0.314[b]	0.035	0.412[b]	0.052	0.461[ab]	0.048	0.634[a]	0.079	**0.003**	**<0.001**

SEM, standard error of the mean; Group 1: ALT <30 IU/L for males, ALT < 20 IU/L for females; group 2: 30 IU/L ≤ ALT < 60 IU/L for males, 20 IU/L ≤ ALT < 40 IU/L for females; group 3: ALT ≥60 IU/L for males, ≥40 IU/L for females. P-value, significance level for comparisons between groups; P-value for trend, significance level for the linear trend across the groups; Groups displaying different letters are significantly different (Tukey HSD test, $p<0.05$).

Figure legends

Figure 1. Representative liver histopathology of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

Liver sections were stained with H&E and sirius red (x200, scale bar: 100 um). The black arrow indicates an inflammatory focus, and the inset shows hepatocyte ballooning.

Figure 2. Relative expression levels of steatosis-related genes in the livers of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

Relative expression levels were determined using RT-PCR. GAPDH was used as the endogenous control. The mean expression level in the B6 mice fed the SD was set at 1. Data are shown as the mean ± SEM (n=5). * $p<0.05$ compared with the B6, SD mice; # $p<0.05$ compared with the db/db, SD mice

Figure 3. Relative abundance of free polyunsaturated fatty acids in the livers (A) and plasma (B) of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or the choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

The relative abundance of each PUFA was measured as described in the Methods section. The mean level seen in the B6 mice fed the SD was set at 1. Data are shown as the mean ± SEM (n=5). * $p<0.05$ compared with the B6, SD mice. # $p<0.05$ compared with the db/db, SD mice

Figure 4. Relative expression levels of ELOVL2 and ELOVL5 in the livers of C57BL/6J (B6) mice fed the standard diet (SD) and db/db mice fed the SD or choline deficient L-amino acid defined high fat diet (CDAHFD) for 4 weeks

Relative expression levels were determined using RT-PCR. GAPDH was used as an endogenous control. The mean expression level of the B6 mice fed the SD was set at 1. Data are shown as the mean ± SEM (n=5). * $p<0.05$ compared with the B6, SD mice; # $p<0.05$ compared with the db/db, SD mice

Figure 5. Relative expression levels of proinflammatory genes in adrenic acid (ADA)-pretreated HepG2 that had been stimulated with rhTNFα

Relative expression levels were determined using RT-PCR. The mRNA levels of TNFα, IL8, and MIP1β were normalized to natural logarithm values. All bars indicate the difference compared with the untreated cells, except for MCP1. No MCP1 mRNA expression was detected in the untreated cells, so the mean expression level of MCP1 in the rhTNFα-treated HepG2 cells was set at 1. β-actin was used as an endogenous control. Data are shown as the mean ± SEM (n=3). *$p<0.05$ compared with the rhTNFα-treated HepG2 cells; n.s.: not significant

Figure 6. Relative expression levels of proinflammatory genes in adrenic acid (ADA)-pretreated HepG2 that had been stimulated with rhIL1β

Relative expression levels were determined using RT-PCR. The mRNA levels of TNFα, IL8, and MIP1β were normalized to natural logarithm values. All bars indicate the difference compared with the untreated cells, except for MCP1. No MCP1 mRNA expression was detected in the untreated cells, so the mean expression of MCP1 in the rhIL1β-treated HepG2 cells was

set at 1. β-actin was used as an endogenous control. Data are shown as the mean ± SEM (n=3). *p<0.05 compared with the rhIL1β-treated HepG2 cells

Figure 7. Relative expression levels of TNFR-1(A), IL1R-1, and IL1R-2 (B) in adrenic acid (ADA)-pretreated HepG2 that had been stimulated with rhTNFα or IL1β

Relative expression levels were determined using RT-PCR. All bars indicate the difference compared with the untreated cells (mean expression set at 1). β-actin was used as an endogenous control. Data are shown as the mean ± SEM (n=3). #p<0.05 compared with the rhIL1β-treated HepG2 cells; n.s.: not significant

Figure 1.

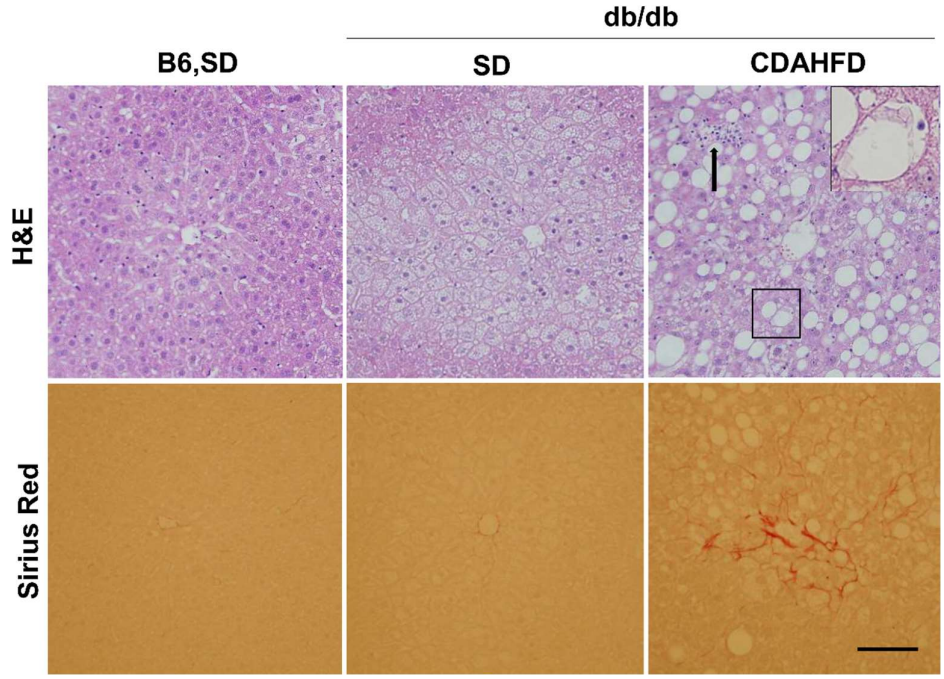


Figure 2.

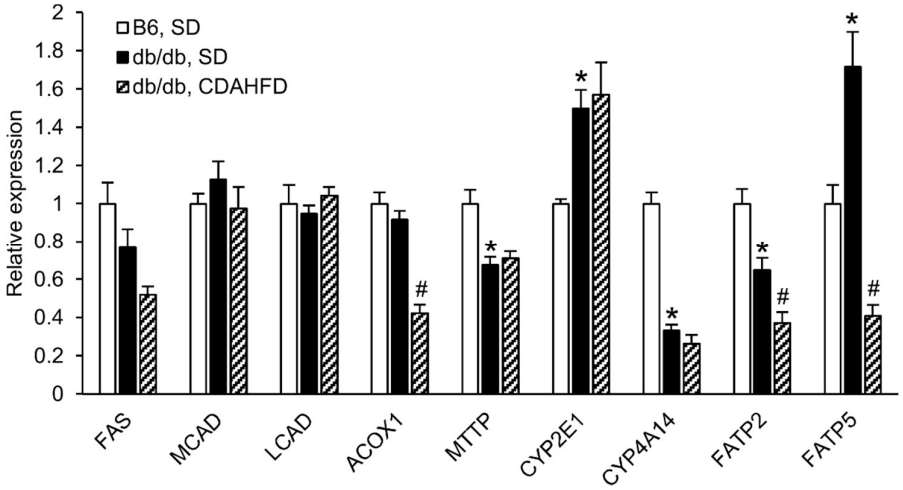

B6, SD
db/db, SD
db/db, CDAHFD
Relative expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FAS
MCAD
LCAD
ACOX1
MTTP
CYP2E1
CYP4A14
FATP2
FATP5
*
#

Figure 3.

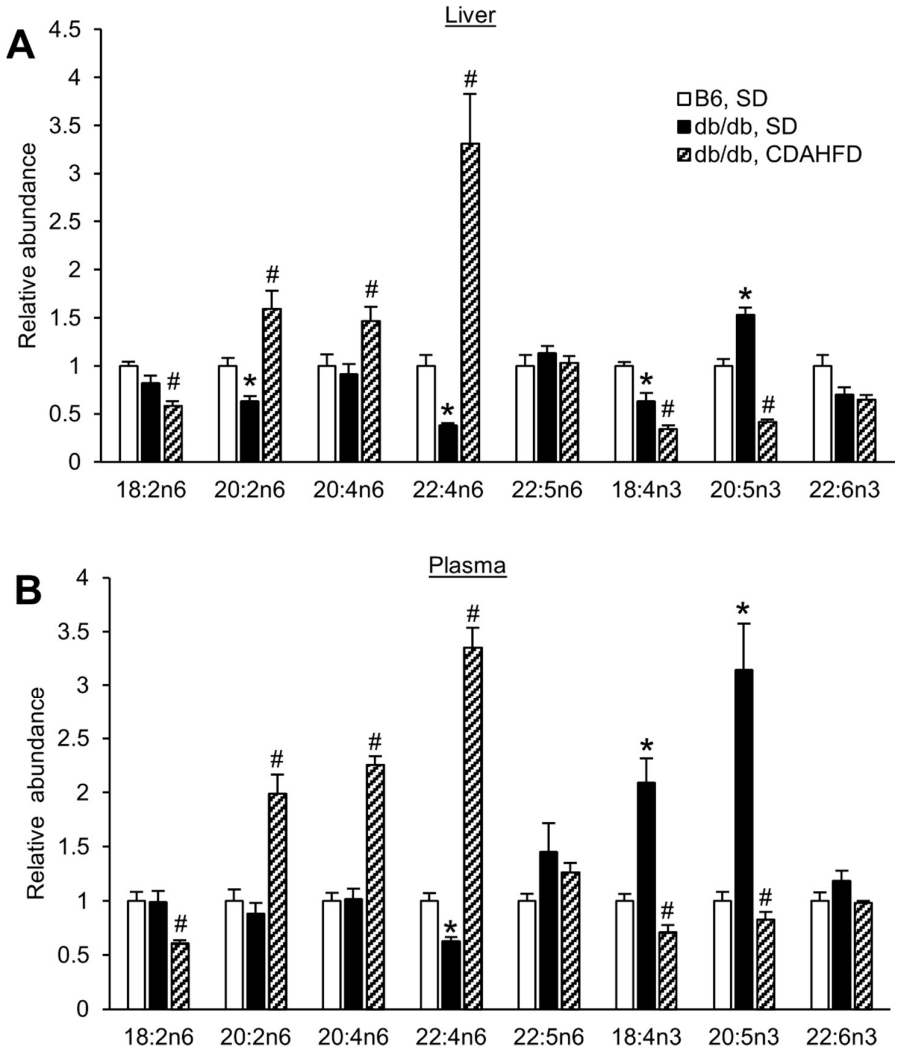

A
Liver
Relative abundance
B6, SD
db/db, SD
db/db, CDAHFD
18:2n6
20:2n6
20:4n6
22:4n6
22:5n6
18:4n3
20:5n3
22:6n3
B
Plasma
Relative abundance
18:2n6
20:2n6
20:4n6
22:4n6
22:5n6
18:4n3
20:5n3
22:6n3

Figure 4.

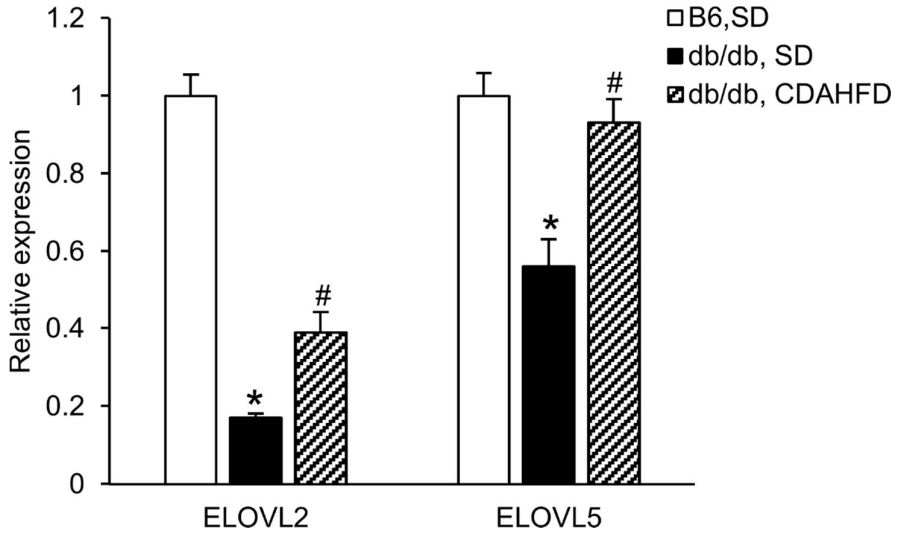

1.2
1
0.8
0.6
0.4
0.2
0
Relative expression
B6,SD
db/db, SD
db/db, CDAHFD
*
#
*
#
ELOVL2
ELOVL5

Figure 5.

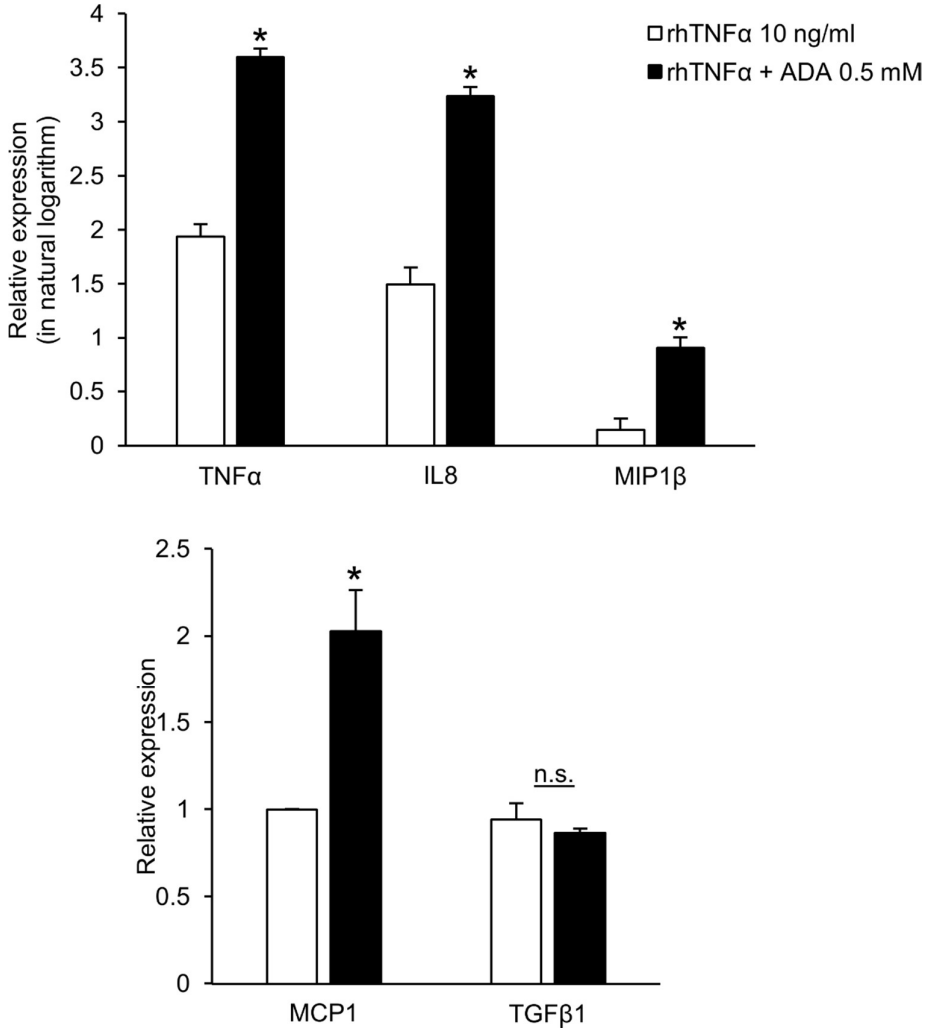

rhTNFα 10 ng/ml
rhTNFα + ADA 0.5 mM
Relative expression
(in natural logarithm)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
TNFα
IL8
MIP1β
Relative expression
2.5
2
1.5
1
0.5
0
*
n.s.
MCP1
TGFβ1

Figure 6.

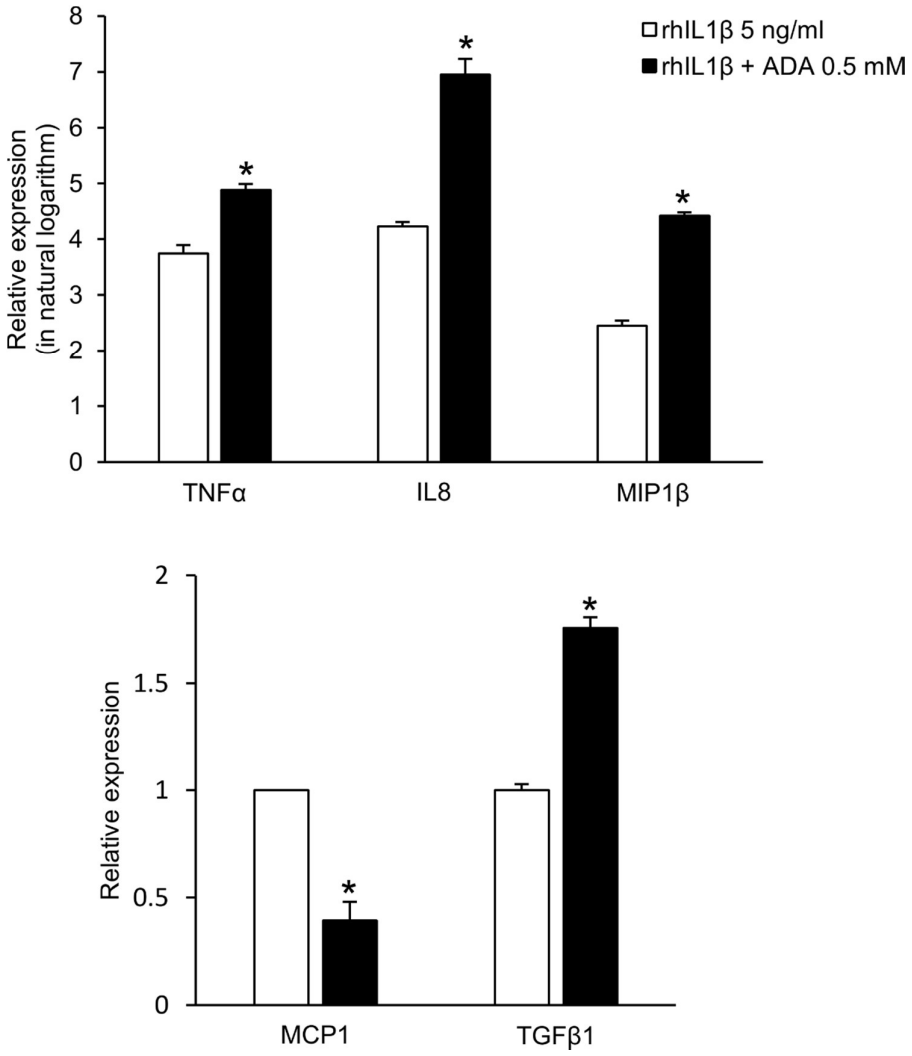
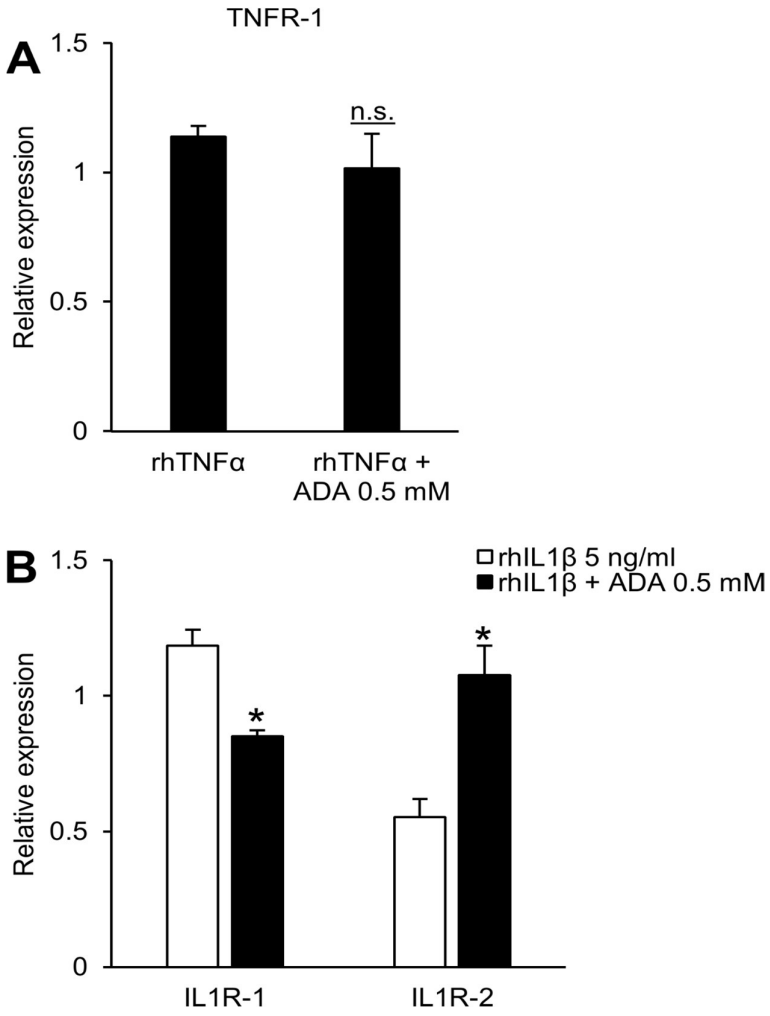

rhIL1β 5 ng/ml
rhIL1β + ADA 0.5 mM
Relative expression (in natural logarithm)
0
1
2
3
4
5
6
7
8
*
*
*
TNFα
IL8
MIP1β
Relative expression
0
0.5
1
1.5
2
*
*
MCP1
TGFβ1

Figure 7.


A
TNFR-1
1.5
1
0.5
0
Relative expression
n.s.
rhTNFα
rhTNFα +
ADA 0.5 mM
B
rhIL1β 5 ng/ml
rhIL1β + ADA 0.5 mM
1.5
1
0.5
0
Relative expression
*
*
IL1R-1
IL1R-2

Higlights

- Lipid analysis of an animal model of non-alcoholic fatty liver disease (NAFLD) identified adrenic acid (22:4n6) as a contributor to disease progression
- Adrenic acid supplementation enhanced cytokine and chemokine gene expression in hepatocytes.
- Lipid analysis of the plasma of human NAFLD patients showed that adrenic acid was associated with higher alanine aminotransferase levels.

Supplementary Data

Supplementary Table S1. Compositions of the diets

	SD	CDAHFD
Carbohydrates	60%	20%
Protein	28%	18%
Total fat	12%	62%
SFA	21%	32%
MUFA	25.3%	35.9%
PUFA	51.2%	32%
Omega 6		
LA	44.4%	28.7%
AA	0.0%	0.3%
Omega 3		
ALA	3.3%	2%
EPA	2.3%	0%
DHA	1.3%	0%
Methionine	0.4%	0.1%
Choline	0.21%	0%

Carbohydrates, protein, and total fat are presented as kcal% values. SFA, MUFA, and PUFA (omega 6 and 3) are shown as percentages of the total fatty acid content. Methionine and choline are presented as gram% values. SD, standard diet; CDAHFD, choline deficient, L-amino acid defined, high-fat diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA,

polyunsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid

Supplementary Table S2. RT-qPCR primer sequences

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
Mouse		
TNFα	5'-tggcccagaccctcacactcag-3'	5'-ggtggtttgctacgacgtgggc-3'
CD68	5'-taccaattcagggtggaag-3'	5'-ctggaccttggttttgttgg-3'
TGFβ1	5'-tgagtggctgtcttttgacg-3'	5'-agtgagcgctgaatcgaaag-3'
COL1α1	5'-tcgagctcagaggcgaaggca-3'	5'-ggtgtgactcgtgcagccgt-3'
FAS	5'-tgattatggccctcagttcc-3'	5'-cagcattgtgtccatgaagg-3'
MCAD	5'-accgaagagttggcgtatgg-3'	5'-cacaggcatttgccccaaag-3'
LCAD	5'-tgcacacatacagacggtgc-3'	5'-catggaagcagaaccggagt-3'
MTTP	5'-agaatgaaggctgcaagctc-3'	5'-gaagcaaggcattcttcagg-3'
ACOX1	5'-gggagtgctacgggttacatg-3'	5'-ccgatatccccaacagtgatg-3'
CYP2E1	5'-tccacaggaaaacgagtgtg-3'	5'-ctttgggtcaacgagaggct-3'
CYP4A14	5'-gtgactggggaatggggaaa-3'	5'-aggctggcctttggtcttttt-3'
ELOVL2	5'-caacatgtttggaccacgag-3'	5'-tgatggtgaggatgaaggtg-3'
ELOVL5	5'-catccttcgcaagaacaacc-3'	5'-tgaggacatggatgaagctg-3'
FATP2	5'-tggggctactttagctttgc-3'	5'-actgaatgaccgtgacgttg-3'
FATP5	5'-tggattccttggctgcttac-3'	5'-atcactgttacgccatgctg-3'
UCP2	5'-tacaaggggttcatgccttc-3'	5'-attggtaggcagccattagg-3'
GPx1	5'-ggacaccaggagaatggcaa-3'	5'-gtaaagagcgggtgagcctt-3'
GAPDH	5'-aaatggtgaaggtcggtgtg-3'	5'-aatctccactttgccactgc-3'
Human		
TNFα	5'-ggcagtcagatcatcttctcgaa-3'	5'-tgaagaggacctgggagtagatg-3'
IL8	5'-ctcttggcagccttcctgatttct-3'	5'-gtttcactggcatcttcactgatt-3'
MIP1β	5'-tcatgctagtagctgccttctg-3'	5'-accacaaagttgcgaggaag-3'
MCP1	5'-agcaagtgtcccaaagaagc-3'	5'-tggaatcctgaacccacttc-3'
TGFβ1	5'-ttgatgtcaccggagttgtg-3'	5'-aacccgttgatgtccacttg-3'
TNFR1	5'-accaagtgccacaaaggaac-3'	5'-gttttctgaagcggtgaagg-3'
IL1R1	5'-gctcatcgtgatgaatgtgg-3'	5'-gccttgtgggtttgttttcc-3'
IL1R2	5'-ggccagcaatacaacatcac-3'	5'-tcccagaaacaccttacacg-3'
β-actin	5'-aaatctggcaccaccttc-3'	5'-tgatctgggtcatcttctcg-3'

Supplemental Table S3. The liver lipid species profile of db/db mice fed SD or CDAHFD after 4 weeks feeding

(NOTE)

CT, control, B6 fed standard diet (SD); SS, simple steatosis, *db/db* mice fed SD; SH, steatohepatitis, *db/db* mice fed choline deficient L-amino acid defined high fat diet (CDAHFD)

The column SS/CT indicates fold change between simple steatosis and control group; SH/SS indicates fold change between steatohepatitis and simple steatosis group.

p-values are based on Student's t-test.

liver	SS/CT	p-value (SS/CT)	SH/SS	p-value (SH/SS)
LPC_14-0 (sn-1)	0.46	0.0017	1.64	0.0917
LPC_14-0 (sn-2)	0.53	0.0013	1.36	0.1368
LPC_15-0 (sn-2)	0.44	0.0175	1.00	0.9888
LPC_16-0e	0.36	0.0771	1.25	0.2866
LPC_16-1 (sn-1)	1.05	0.8185	0.54	0.0272
LPC_16-1 (sn-2)	0.94	0.8021	0.69	0.2162
LPC_16-0 (sn-1)	0.84	0.4595	0.84	0.3834
LPC_16-0 (sn-2)	1.06	0.6959	1.04	0.7433
LPC_17-1 (sn-1)	0.99	0.9785	0.84	0.6476
LPC_17-1 (sn-2)	0.72	0.1329	0.67	0.1234
LPC_17-0 (sn-1)	1.17	0.6320	0.51	0.1286
LPC_17-0 (sn-2)	1.20	0.2869	0.72	0.0816
LPC_18-3 (sn-1)	0.44	0.0410	1.02	0.9380
LPC_18-3 (sn-2)	1.57	0.1511	0.45	0.0447
LPC_18-2 (sn-1)	1.12	0.5371	0.65	0.0312
LPC_18-2 (sn-2)	1.13	0.5984	0.49	0.0339
LPC_18-1 (sn-1)	0.81	0.2527	1.17	0.2730
LPC_18-1 (sn-2)	1.16	0.4990	0.74	0.1902
LPC_18-0 (sn-1)	1.81	0.0820	0.54	0.0736
LPC_18-0 (sn-2)	2.34	0.0111	0.77	0.1634
LPC_19-0 (sn-1)	0.69	0.4146	0.27	0.0481
LPC_19-0 (sn-2)	0.95	0.8318	0.38	0.0400
LPC_20-5 (sn-1)	4.23	0.0274	0.16	0.0204
LPC_20-4 (sn-1)	0.81	0.3493	2.32	<0.001
LPC_20-3 (sn-1)	3.41	0.0025	0.91	0.4968
LPC_20-3 (sn-2)	2.90	0.0237	0.91	0.7474
LPC_20-2 (sn-1)	0.89	0.5740	2.08	0.0028
LPC_20-2 (sn-2)	1.35	0.2805	0.97	0.8946
LPC_20-1 (sn-1)	0.44	0.0243	1.11	0.6658
LPC_20-1 (sn-2)	0.96	0.8900	0.60	0.1368
LPC_20-0 (sn-1)	1.08	0.8784	0.21	0.1255
LPC_20-0 (sn-2)	1.18	0.5106	0.57	0.0882
LPC_22-6 (sn-1)	1.10	0.6990	1.25	0.1637
LPC_22-4 (sn-1)	0.58	0.1187	5.72	0.0026
LPC_22-0 (sn-1)	1.30	0.3455	0.77	0.5364
LPC_22-0 (sn-2)	0.61	0.0029	0.81	0.3881
PC_14-0_16-1	1.44	0.0059	1.12	0.2581
PC_16-0_14-0	0.78	0.0238	1.27	0.0032
PC_15-0_16-1	0.56	0.0018	0.49	<0.001
PC_16-0p_16-0	0.55	<0.001	0.85	0.3666
PC_16-0_15-0	0.67	0.1257	0.47	0.0946
PC_16-0e_16-0	0.41	<0.001	1.53	0.0069
PC_14-0_18-2 PC_16-1_16-1	0.41	<0.001	0.81	0.0745
PC_14_0-18_1 PC_16-0_16-1	1.01	0.8597	0.65	<0.001
PC_16-0_16-0	0.57	<0.001	1.26	0.0211
PC_15-0_18-2	0.35	<0.001	0.75	0.0429
PC_16-0e_18-2	0.68	0.0078	0.84	0.1428
PC_16-1e_18-1	0.57	<0.001	0.94	0.7417
PC_15-0_18-1 PC_16-0_17-1	0.63	0.0049	0.86	0.1531
PC_18-1e_16-0 PC_18-0e_16-1	0.54	<0.001	1.64	0.0047
PC_17-0_16-0 PC_18-0_15-0	0.49	<0.001	1.73	0.0044
PC_18-0e_16-0	0.37	0.0016	1.72	0.0590
PC_14-0_20-5	0.93	0.7267	0.49	0.0311
PC_16-1_18-3 PC_14-0_20-4	0.40	<0.001	2.00	0.0041
PC_14-0_20-3	0.43	<0.001	1.12	0.1959
PC_16-1_18-2 PC_16-0_18-3	0.43	<0.001	1.12	0.1959
PC_16-0_18-2 PC_16-1_18-1	0.89	0.1920	0.75	0.0124
PC_16-0_18-1	0.79	0.0365	0.95	0.5280
PC_16-0_18-0	0.77	0.0234	1.13	0.1990
PC_15-0_20-5	1.17	0.4596	0.13	0.0070
PC_16-0e_20-5	0.33	<0.001	2.52	<0.001
PC_16-0p_20-4	0.33	<0.001	2.52	<0.001
PC_15-0_20-4	0.40	<0.001	1.38	0.0157

PC_16-1e_20-3	0.46	<0.001	2.29	<0.001
PC_17-1_18-2	0.58	0.0013	0.47	0.0017
PC_18-1e_18-2	0.97	0.8703	1.04	0.8770
PC_18-2e_18-1	0.49	0.0016	0.30	<0.001
PC_17-1_18-1 PC_17-0_18-2	0.88	0.2905	0.49	0.0036
PC_18-0p_18-1 PC_18-1e_18-1	0.26	0.0017	1.03	0.9437
PC_16-0e_20-2	0.37	0.2103	0.91	0.7490
PC_17-0_18-1 PC_17-1_18-0 PC_16-0_19-1	0.56	<0.001	0.90	0.3069
PC_16-1_20-5	1.39	0.2272	0.19	0.0141
PC_14-0_22-6	0.56	0.0014	1.07	0.5125
PC_18-2_18-3	0.68	0.0090	0.70	0.0544
PC_14-0_22-5 PC_16-1_20-4 PC_16-0_20-5	2.34	0.0080	0.24	0.0025
PC_18-2_18-2 PC_18-1_18-3	0.80	0.0843	0.44	<0.001
PC_16-0_20-4 PC_16-1_20-3	0.69	0.0019	2.23	<0.001
PC_18-1_18-2 PC_16-0_20-3 PC_18-0_18-3	1.09	0.4104	0.80	0.0772
PC_18-1_18-1 PC_18-0_18-2	1.41	0.0113	0.56	0.0014
PC_18-0_18-1	1.42	0.0537	0.67	0.0376
PC_16-0p_22-6	0.49	<0.001	0.95	0.6726
PC_18-0_18-0	0.82	0.3083	2.15	<0.001
PC_15-0_22-6	0.31	<0.001	1.37	0.0655
PC_18-1e_20-5	0.59	0.0018	0.98	0.8211
PC_16-0e_22-6 PC	0.60	0.0019	0.98	0.8316
PC_17-0_20-5 PC_17-1_20-4	1.22	0.0920	0.58	0.0032
PC_16-0e_22-5 PC_18-0e_20-5	0.55	0.0054	2.09	0.0039
PC_18-0p_20-4	0.39	0.0041	2.25	0.0048
PC_17-0_20-4	0.64	0.0020	1.63	0.0053
PC_18-1e_20-3	0.45	0.0016	2.85	0.0092
PC_18-0e_20-4	0.28	0.0008	2.64	<0.001
PC_17-0_20-3 PC_19-1_18-2	1.45	0.0503	0.37	0.0043
PC_19-1_18-1 PC_19-0_18-2	0.61	0.0044	0.27	0.0026
PC_19-0_18-1 PC_18-0_19-1	0.54	0.0064	0.46	0.0154
PC_18-2_20-5 PC_16-1_22-6	0.97	0.8695	0.59	0.0903
PC_18-2_20-4 PC_16-0_22-6	1.03	0.7167	0.94	0.5016
PC_18-1_20-4	0.73	0.1401	2.10	<0.001
PC_18-0_20-5	1.58	0.1308	1.12	0.5183
PC_18-1_20-3	2.59	0.0048	0.55	0.0118
PC_18-0_20-4	0.84	0.1418	1.88	<0.001
PC_18-1_20-2 PC_18-0_20-3	3.05	0.0028	0.66	0.0318
PC_16-0_22-2	2.87	0.0014	0.67	0.0180
PC_18-0_20-2	0.97	0.8608	0.70	0.1464
PC_20-0_18-1	0.92	0.4866	0.64	0.0084
PC_18-1e_22-6	0.69	0.0324	1.10	0.5450
PC_17-0_22-6	0.74	0.0216	0.97	0.8378
PC_18-1e_22-5	0.26	0.0833	2.77	0.0792
PC_18-0p_22-5	0.27	0.0018	0.91	0.3447
PC_19-0_20-3	0.85	0.4409	0.59	0.1290
PC_20-4_20-4	1.01	0.9334	0.82	0.2035
PC_20-3_20-4	1.46	0.0065	0.70	0.0314
PC_18-1_22-6	1.04	0.7674	1.09	0.5197
PC_20-2_20-4 PC_18-1_22-5	0.93	0.4807	1.24	0.0578
PC_18-0_22-6	1.25	0.0565	0.91	0.3724
PC_18-0_22-5	1.70	0.0029	0.86	0.2875
PC_20-1_20-3 PC_18-0_22-4	0.59	0.0970	4.99	<0.001
PC_18-1_22-0	0.74	0.1137	0.26	0.0066
PC_19-0_22-6	0.41	<0.001	0.51	0.0063
LPE_16-0 (sn-1)	1.10	0.7516	0.60	0.1293
LPE_16-0 (sn-2)	1.25	0.2030	0.79	0.1001
LPE_17-0 (sn-1)	1.49	0.2716	0.36	0.0595
LPE_17-0 (sn-2)	1.07	0.7519	0.52	0.0328
LPE_18-2 (sn-1)	1.22	0.2419	0.36	0.0069
LPE_18-2 (sn-2)	1.23	0.3823	0.36	0.0219
LPE_18-1 (sn-1)	1.25	0.3965	0.44	0.0412
LPE_18-1 (sn-2)	1.56	0.0515	0.55	0.0246
LPE_18-0 (sn-1)	1.61	0.2006	0.53	0.1217
LPE_18-0 (sn-2)	1.48	0.0540	0.76	0.1124
LPE_20-5 (sn-1)	3.49	0.0077	0.10	0.0037
LPE_20-4 (sn-1)	0.72	0.0464	1.30	0.0022
LPE_20-3 (sn-1)	2.40	0.0013	0.63	0.0113
LPE_20-3 (sn-2)	2.68	0.0527	0.60	0.1896
LPE_20-1 (sn-2)	0.56	0.1053	0.60	0.0949
LPE_20-0 (sn-2)	0.75	0.3447	0.49	0.0493
LPE_22-6 (sn-1)	1.09	0.6409	1.01	0.9688
PE_14-0_18-2	0.45	0.0019	0.46	0.0052
PE_16-0_16-1 PE_14-0_18-1	1.16	0.0772	0.31	<0.001

PE_16-0_16-0	0.85	0.4312	1.03	0.8833
PE_16_1_18_2	0.50	0.0145	0.53	0.1290
PE_15-0_18-2	0.39	0.0067	0.57	0.1568
PE_16-0p_18-1	0.29	<0.001	2.43	0.0538
PE_15-0_18-1	0.92	0.7260	0.23	0.0167
PE_16-0e_18-1	0.31	0.0025	0.24	<0.001
PE_16-1_18-2	0.67	0.0029	0.33	<0.001
PE_16-0_18-3	0.74	0.0131	0.73	0.0229
PE_16-1_18-1 PE_16-0_18-2	1.06	0.5197	0.46	<0.001
PE_16-0_18-1	1.02	0.8091	0.50	<0.001
PE_16-0_18-0	1.77	0.2440	0.62	0.2692
PE_16-0p_20-4	0.36	<0.001	3.50	<0.001
PE_16-0p_20-3 PE_16-0e_20-4	0.40	<0.001	1.55	0.2302
PE_17-1_18-2	0.87	0.2021	0.34	<0.001
PE_18-1p_18-1 PE_18-0p_18-2 PE_18-0e_18-3	0.53	0.0358	0.65	0.0986
PE_17-0_18-2	0.77	0.1137	0.40	0.0080
PE_18-0e_18-2	0.45	0.0044	0.41	0.0045
PE_18-0p_18-1	0.67	0.5804	1.11	0.5634
PE_17-0_18-1	0.58	0.0031	0.47	0.0053
PE_18-0e_18-1	0.68	0.7477	1.30	0.6214
PE_16-1_20-5	4.52	0.0051	0.06	0.0025
PE_18-2_18-3	1.87	0.3810	0.36	0.2502
PE_16-0_20-5	3.03	0.0010	0.21	<0.001
PE_18-2_18-2	1.02	0.8515	0.33	0.0010
PE_16-0_20-4	0.89	0.0903	1.35	0.0092
PE_18-1_18-2	0.94	0.5283	0.35	<0.001
PE_18-1_18-1 PE_18-0_18-2	1.13	0.3019	0.44	0.0023
PE_16-0_20-1 PE_18-0_18-1	1.17	0.0820	0.53	<0.001
PE_16-0p_22-6	0.58	<0.001	1.37	0.0185
PE_18-0p_20-5 PE_18-1p_20-4 PE_16-0e_22-6	0.78	0.1129	1.04	0.7890
PE_17-1_20-4	1.73	0.0014	0.49	<0.001
PE_18-1p_20-3 PE_16-0p_22-4	0.99	0.9461	0.58	0.0629
PE_18-0p_20-4	0.51	0.0013	2.75	<0.001
PE_17-0_20-4	0.51	<0.001	1.18	0.2467
PE_18-1e_20-3	0.34	0.0447	1.46	0.4682
PE_18-0e_20-4 PE_20-0e_18-4 PE_20-1e_18-3	0.19	<0.001	2.72	0.0643
PE_19-0_18-2	0.37	0.0782	0.17	0.1044
PE_20-0e_18-1	0.13	0.0205	0.64	0.2942
PE_16-1_22-6 PE_18-2_20-5	1.16	0.2022	0.54	0.0039
PE_18-2_20-4 PE_18-1_20-5	1.26	0.0112	0.72	<0.001
PE_16-0_22-6 PE_16-1_22-5 PE_20-2_18-4	1.26	0.0112	0.72	<0.001
PE_18-1_20-4	1.05	0.4509	1.24	0.0170
PE_18-0_20-5	2.24	0.0021	0.19	<0.001
PE_16-0_22-4	1.07	0.2481	1.31	0.0081
PE_18-1_20-3 PE_18-2_20-2	2.14	<0.001	0.34	<0.001
PE_18-0_20-4	0.65	<0.001	1.27	0.0023
PE_20-1_18-2	0.67	0.0152	1.37	<0.001
PE_18-0_20-3	1.32	0.0151	0.62	0.0035
PE_18-1_20-1	1.43	0.0098	0.58	0.0030
PE_20-0_18-2	0.62	0.0150	0.75	0.2057
PE_18-0_20-1	0.63	0.5859	1.07	0.9132
PE_17-1_22-6	1.30	0.0880	0.74	0.0768
PE_18-0p_22-6 PE_18-1p_22-5	0.57	0.0109	1.18	0.1664
PE_17-0_22-6	0.58	<0.001	1.05	0.6105
PE_18-0p_22-5 PE_18-1p_22-4	0.59	0.0116	0.80	0.0780
PE_17-0_22-5	0.72	0.0618	0.25	0.0054
PE_19-0_20-4	0.17	<0.001	0.65	0.0567
PE_20-0e_20-4 PE_18-0e_22-4	0.52	<0.001	0.96	0.6488
PE_18-1_22-6	0.98	0.8579	1.01	0.8662
PE_18-1_22-5	0.83	0.0389	1.10	0.1481
PE_18-0_22-6	0.89	0.2337	1.00	0.9682
PE_18-0_22-5	1.11	0.2801	0.78	0.0502
PE_18-0_22-4 PE_20-0_20-4	0.15	0.0137	2.09	0.0344
PE_22-2_18-1 PE_22-1_18-2	0.65	0.4589	0.30	0.0756
PE_20-2_22-6	0.44	<0.001	2.20	<0.001
PE_20-1_22-6	0.22	<0.001	1.86	0.0461
PE_22-1_20-4	0.37	0.0690	1.78	0.1179
FA_12-0_Lauric acid	1.00	0.9863	1.04	0.7830
FA_14-1 (n-5)_Myristoleic acid	0.51	0.0023	1.04	0.8525
FA_14-0_Myristic acid	0.55	0.0163	1.48	0.0337
FA_16-1 (n-7)_Palmitoleic acid	1.21	0.3592	0.61	0.0371
FA_16-0_Palmitic acid	1.12	0.6273	1.28	0.0819
FA_17-1 (n-7)_cis-10-Heptadecanoic acid	0.78	0.1850	0.80	0.2610
FA_17-0_Margaric acid	0.97	0.8856	0.70	0.1542

FA_18-4 (n-3)_Stearidonic acid	0.63	0.0074	0.54	0.0197
FA_18-3 (n-3)_Alpha-linolenic acid_(n-6)_gamma-Linolenic acic	0.83	0.1214	0.63	0.0198
FA_18-2 (n-6)_Linoleic acid	0.82	0.0871	0.71	0.0355
FA_18-1 (n-9)c_Oleic acid_(n-7)c_Vaccenic acid	0.78	0.0568	1.09	0.5278
FA_18-1 (n-9)t_Elaidic acid_(n-7)t_Vaccenic acid	0.83	0.1841	1.00	0.9959
FA_18-0_Stearic acid	1.24	0.0913	1.07	0.6640
FA_20-5 (n-3)_Eicosapentaenoic acid	1.53	<0.001	0.28	<0.001
FA_20-4 (n-6)_Arachidonic acid	0.91	0.5975	1.61	0.0159
FA_20-3 (n-6)_Dihomo-gamma-linolenic acid_(n-9)_Mead acic	1.67	0.0057	1.40	0.0224
FA_C20-2 (n-6)_cis-11-14-Eicosadienoic acid	0.63	0.0080	2.53	0.0047
FA_20-1 (n-9)_cis-11-Eicosenoic acid	0.30	0.0135	1.99	0.0130
FA_20-0_Arachidic acid	0.62	0.0204	2.03	0.0148
FA_22-6 (n-3)_Docosahexaenoic acid	0.70	0.0687	0.93	0.5756
FA_22-5 (n-6)_Docosapentaenoic acid	1.13	0.3585	0.91	0.3481
FA_22-4 (n-6)_Docosatetraenoic acid	0.38	0.0047	8.67	0.0045
FA_22-1 (n-9)_Erucic acid	0.68	0.4282	2.07	0.3508
FA_22-0_Behenic acid	0.89	0.2305	1.40	0.0019
FA_23-0_Tricosanoic acid	0.80	0.3332	1.09	0.5940
FA_24-1 (n-9)_Nervonic acid	0.44	0.0059	1.32	0.1437
AC_16-0	0.73	0.1597	0.83	0.5071
AC_16-1	1.00	0.9847	0.47	0.0061
AC_18-0	0.48	0.0175	0.90	0.5932
AC_18-1	0.76	0.3884	1.04	0.8857
AC_18-2	0.62	0.1276	0.62	0.2159

Supplemental Table S4. The plasma lipid species profile of db/db mice fed SD or CDAHFD after 4 weeks feeding

(NOTE)

CT, control, B6 fed standard diet (SD); SS, simple steatosis, *db/db* mice fed SD; SH, steatohepatitis, *db/db* mice fed choline deficient L-amino acid defined high fat diet (CDAHFD)

The column SS/CT indicates fold change between simple steatosis and control group; SH/SS indicates fold change between steatohepatitis and simple steatosis group.

p-values are based on Student's t-test.

plasma	SS/CT	p-value (SS/CT)	SH/SS	p-value (SH/SS)
LPC_14-0 (sn-1)	0.43	< 0.001	1.96	<0.001
LPC_14-0 (sn-2)	0.45	< 0.001	1.84	0.0041
LPC_16-0p	0.98	0.9398	1.42	0.0224
LPC_15-0 (sn-1)	0.41	< 0.001	1.18	0.3467
LPC_15-0 (sn-2)	0.43	< 0.001	1.28	0.1181
LPC_16-0e	0.89	0.4494	1.59	0.0341
LPC_16-1 (sn-1)	0.54	0.0014	1.51	0.0320
LPC_16-1 (sn-2)	0.52	< 0.001	1.58	0.0591
LPC_16-0 (sn-1)	0.84	0.0653	1.77	0.0026
LPC_16-0 (sn-2)	0.81	0.0292	1.72	<0.001
LPC_17-1 (sn-1)	0.40	0.0028	1.88	0.0502
LPC_17-1 (sn-2)	0.41	<0.001	1.76	0.0491
LPC_17-0 (sn-1)	1.12	0.3276	1.11	0.3995
LPC_17-0 (sn-2)	1.00	0.9690	1.34	0.0267
LPC_18-3 (sn-1)	0.46	0.0058	1.96	0.0029
LPC_18-3 (sn-2)	0.45	<0.001	1.75	0.0701
LPC_18-2 (sn-1)	0.79	0.1074	1.36	0.0429
LPC_18-2 (sn-2)	0.88	0.0862	1.13	0.3477
LPC_18-1 (sn-1)	0.58	0.0022	2.31	0.0067
LPC_18-1 (sn-2)	0.63	0.0034	2.15	0.0252
LPC_18-0 (sn-1)	1.73	<0.001	1.59	0.0018
LPC_18-0 (sn-2)	1.64	0.0063	1.59	<0.001
LPC_19-0 (sn-1)	0.86	0.2527	0.65	0.0186
LPC_19-0 (sn-2)	0.99	0.9574	0.65	0.0045
LPC_20-5 (sn-1)	2.39	0.0423	0.31	0.0253
LPC_20-5 (sn-2)	2.80	0.0232	0.23	0.0129
LPC_20-4 (sn-1)	0.48	0.0058	4.71	<0.001
LPC_20-4 (sn-2)	0.55	0.0028	3.74	0.0053
LPC_20-3 (sn-1)	1.93	0.0045	1.92	<0.001
LPC_20-3 (sn-2)	2.35	0.0109	1.54	0.0212
LPC_20-2 (sn-1)	0.62	0.0069	3.72	0.0033
LPC_20-2 (sn-2)	0.90	0.4786	2.47	0.0200
LPC_20-1 (sn-1)	0.47	<0.001	1.16	0.5204
LPC_20-1 (sn-2)	0.47	<0.001	1.37	0.1141
LPC_20-0 (sn-1)	1.05	0.7820	1.01	0.9581
LPC_20-0 (sn-2)	0.88	0.1419	1.33	0.0949
LPC_22-6 (sn-1)	0.78	0.0716	3.02	0.0011
LPC_22-6 (sn-2)	0.88	0.3909	2.41	0.0091
LPC_22-4 (sn-1)	0.56	0.0474	9.36	<0.001
LPC_22-4 (sn-2)	0.50	0.0406	7.94	0.0089
LPC_22-0 (sn-1)	0.93	0.6823	1.42	0.0115
LPC_22-0 (sn-2)	0.77	0.0760	1.65	0.0074
PC_14-0_16-1	1.15	0.0414	1.80	<0.001
PC_16-0_14-0	0.68	<0.001	1.82	0.0012
PC_15-0_16-1	0.81	0.1643	0.99	0.9458
PC_16-0p_16-0	0.42	0.0032	0.39	<0.001
PC_16-0_15-0	0.86	0.7326	0.42	0.1696
PC_16-0e_16-0	0.30	0.0001	1.21	0.5624
PC_14-0_18-2 PC_16-1_16-1	0.43	<0.001	2.89	0.0071
PC_14_0-18_1 PC_16-0_16-1	1.04	0.5920	1.79	0.0085
PC_16-0_16-0	0.60	<0.001	1.31	0.0565
PC_15-0_18-2	0.47	<0.001	1.71	0.0026
PC_16-0e_18-2	0.77	0.0113	0.47	<0.001
PC_16-1e_18-1	0.66	<0.001	0.91	0.2808
PC_15-0_18-1 PC_16-0_17-1	0.58	<0.001	1.81	<0.001
PC_18-1e_16-0 PC_18-0e_16-1	0.48	<0.001	1.65	0.2089
PC_17-0_16-0 PC_18-0_15-0	0.46	<0.001	1.71	0.1739
PC_18-0e_16-0	0.30	0.0487	2.68	0.0717
PC_14-0_20-5	1.14	0.5839	1.01	0.9772
PC_16-1_18-3 PC_14-0_20-4	0.44	<0.001	4.73	0.0014
PC_14-0_20-3	0.74	0.1557	2.11	0.0029
PC_16-1_18-2 PC_16-0_18-3	0.42	0.0100	3.67	0.0022
PC_16-0_18-2 PC_16-1_18-1	1.02	0.7680	1.02	0.7072
PC_16-0_18-1	0.77	0.0027	1.80	<0.001
PC_16-0_18-0	1.31	0.0177	1.59	0.0099
PC_15-0_20-5	1.41	0.0940	0.47	0.0137
PC_16-0e_20-5	0.72	0.0390	1.37	0.0641

PC_16-0p_20-4	0.55	0.1938	1.76	0.2554
PC_15-0_20-4	0.47	<0.001	2.71	0.0034
PC_16-1e_20-3	0.55	<0.001	1.61	<0.001
PC_17-1_18-2	0.68	0.0014	1.12	0.3105
PC_18-1e_18-2	0.90	0.3616	0.46	0.0035
PC_18-2e_18-1	0.89	0.7220	0.57	0.1463
PC_17-1_18-1 PC_17-0_18-2	1.18	0.1648	0.73	0.0346
PC_18-0p_18-1 PC_18-1e_18-1	0.64	0.0021	0.94	0.5554
PC_16-0e_20-2	0.31	0.1128	1.20	0.6576
PC_17-0_18-1 PC_17-1_18-0 PC_16-0_19-1	0.54	0.0236	2.33	0.0029
PC_16-1_20-5	1.46	0.1902	1.68	0.2122
PC_14-0_22-6	0.63	0.0067	2.63	0.0255
PC_18-2_18-3	0.73	0.0494	2.44	0.0023
PC_14-0_22-5 PC_16-1_20-4 PC_16-0_20-5	2.41	0.0048	0.47	0.0068
PC_18-2_18-2 PC_18-1_18-3	0.94	0.5245	1.02	0.8082
PC_16-0_20-4 PC_16-1_20-3	0.61	<0.001	2.64	<0.001
PC_18-1_18-2 PC_16-0_20-3 PC_18-0_18-3	1.11	0.0121	1.21	0.0029
PC_18-1_18-1 PC_18-0_18-2	1.45	0.0030	0.87	0.0684
PC_18-0_18-1	1.62	0.0019	1.75	<0.001
PC_16-0p_22-6	0.89	0.2612	0.63	0.0120
PC_18-0_18-0	2.26	0.3962	1.13	0.8264
PC_15-0_22-6	0.37	<0.001	2.99	0.0010
PC_18-1e_20-5	0.12	<0.001	6.62	<0.001
PC_16-0e_22-6 PC	0.77	0.0011	1.01	0.9196
PC_17-0_20-5 PC_17-1_20-4	1.30	0.0665	1.03	0.7572
PC_16-0e_22-5 PC_18-0e_20-5	0.78	0.3764	0.83	0.3800
PC_18-0p_20-4	0.51	0.1366	1.82	0.4225
PC_17-0_20-4	0.57	<0.001	2.89	<0.001
PC_18-1e_20-3	0.40	<0.001	2.60	0.0885
PC_18-0e_20-4	0.88	0.7355	1.71	0.1384
PC_17-0_20-3 PC_19-1_18-2	1.42	0.0050	0.37	<0.001
PC_19-1_18-1 PC_19-0_18-2	1.22	0.0517	0.51	0.0015
PC_19-0_18-1 PC_18-0_19-1	0.94	0.4746	1.06	0.5853
PC_18-2_20-5 PC_16-1_22-6	0.92	0.5357	1.27	0.1657
PC_18-2_20-4 PC_16-0_22-6	1.02	0.7694	1.50	<0.001
PC_18-1_20-4	0.58	0.0535	3.47	<0.001
PC_18-0_20-5	2.59	0.0056	0.84	0.4069
PC_18-1_20-3	2.71	<0.001	0.71	0.0086
PC_18-0_20-4	0.75	0.0194	2.93	<0.001
PC_18-1_20-2 PC_18-0_20-3	2.56	<0.001	1.62	<0.001
PC_16-0_22-2	6.52	<0.001	1.18	0.0379
PC_18-0_20-2	1.32	0.0019	1.53	<0.001
PC_20-0_18-1	0.93	0.8804	3.90	0.0016
PC_18-1e_22-6	0.32	0.0098	2.06	0.0935
PC_17-0_22-6	0.74	0.0175	1.71	<0.001
PC_18-1e_22-5	0.41	0.0077	0.31	<0.001
PC_18-0p_22-5	0.41	0.0019	0.93	0.8809
PC_19-0_20-3	1.01	0.9921	1.17	0.8536
PC_20-4_20-4	0.92	0.2854	1.66	<0.001
PC_20-3_20-4	1.52	0.0033	1.10	0.2431
PC_18-1_22-6	0.78	0.0809	1.53	0.0052
PC_20-2_20-4 PC_18-1_22-5	0.89	0.3704	2.06	<0.001
PC_18-0_22-6	1.31	0.0252	1.33	0.0069
PC_18-0_22-5	1.68	0.0258	1.76	0.0018
PC_20-1_20-3 PC_18-0_22-4	0.71	0.3163	10.99	<0.001
PC_18-1_22-0	0.79	0.2284	0.45	0.0305
PC_19-0_22-6	0.53	<0.001	1.01	0.9398
LPE_16-0 (sn-1)	0.80	0.0935	1.89	0.0029
LPE_16-0 (sn-2)	0.79	0.1206	1.73	0.0020
LPE_17-0 (sn-1)	0.40	<0.001	2.89	0.0030
LPE_17-0 (sn-2)	0.49	0.0196	2.57	0.0775
LPE_18-2 (sn-1)	0.92	0.7112	1.09	0.6410
LPE_18-2 (sn-2)	1.05	0.6803	0.95	0.7037
LPE_18-1 (sn-1)	0.78	0.0652	1.46	0.0335
LPE_18-1 (sn-2)	0.73	0.0090	1.68	0.0047
LPE_18-0 (sn-1)	1.11	0.2531	1.89	0.0055
LPE_18-0 (sn-2)	1.02	0.8329	1.77	<0.001
LPE_20-5 (sn-1)	3.92	0.0199	0.18	0.0152
LPE_20-5 (sn-2)	6.37	0.0014	0.26	0.0017
LPE_20-4 (sn-1)	0.75	0.1493	4.06	<0.001
LPE_20-4 (sn-2)	1.16	0.0826	2.76	0.0017
LPE_20-3 (sn-1)	1.99	0.0093	2.51	0.0165
LPE_20-3 (sn-2)	1.38	0.3213	1.40	0.2568
LPE_22-6 (sn-1)	0.76	0.0572	3.06	<0.001
LPE_22-6 (sn-2)	0.87	0.3358	2.83	0.0031
PE_16-0_16-1 PE_14-0_18-1	1.07	0.8027	0.43	<0.001
PE_16-0_16-0	1.84	0.0804	0.52	0.0725
PE_16_1_18_2	0.43	0.0022	0.81	0.4643

PE_16-0p_18-1	0.76	0.3248	0.47	0.1103
PE_16-0e_18-1	0.52	<0.001	0.66	0.0443
PE_16-1_18-2	0.33	<0.001	0.76	0.3601
PE_16-0_18-3	0.39	0.0162	1.17	0.2189
PE_16-1_18-1 PE_16-0_18-2	0.63	0.0065	0.80	0.1912
PE_16-0_18-1	0.86	0.2931	0.38	0.0075
PE_16-0_18-0	1.10	0.7555	1.41	0.1681
PE_16-0p_20-5	0.91	0.8163	0.18	0.0740
PE_16-0p_20-4	0.26	<0.001	1.83	0.0096
PE_16-0p_20-3 PE_16-0e_20-4	0.53	0.1335	0.93	0.6705
PE_17-1_18-2	0.69	0.3654	0.54	0.2136
PE_18-1p_18-1 PE_18-0p_18-2 PE_18-0e_18-3	0.77	0.2658	0.54	0.1053
PE_17-0_18-2	0.62	0.0352	0.43	0.0301
PE_18-0e_18-2	0.68	0.0057	0.64	0.0153
PE_18-0p_18-1	0.70	0.6170	1.01	0.9803
PE_17-0_18-1	0.45	0.1885	2.38	0.4077
PE_18-0e_18-1	0.78	0.4318	0.80	0.1370
PE_16-1_20-5	1.67	0.1138	0.24	0.0157
PE_18-2_18-3	1.16	0.8537	0.58	0.5866
PE_16-0_20-5	1.78	0.0190	0.41	0.0049
PE_18-2_18-2	0.64	0.0185	0.41	0.0131
PE_16-0_20-4	0.58	0.0025	1.87	0.0088
PE_18-1_18-2	0.40	0.5667	0.25	0.4354
PE_18-1_18-1 PE_18-0_18-2	0.60	0.0136	0.64	0.0263
PE_16-0_20-1 PE_18-0_18-1	0.66	0.0119	0.78	0.1172
PE_16-0p_22-6	0.65	<0.001	0.61	0.0023
PE_18-0p_20-5 PE_18-1p_20-4 PE_16-0e_22-6	0.61	<0.001	0.58	0.0027
PE_17-1_20-4	0.93	0.6420	0.70	0.0249
PE_18-1p_20-3 PE_16-0p_22-4	0.84	0.2022	0.24	<0.001
PE_18-0p_20-4	0.52	0.0050	1.53	0.0473
PE_17-0_20-4	0.27	0.0012	1.92	0.0147
PE_18-1e_20-3	1.00	0.9919	1.37	0.0237
PE_18-0e_20-4 PE_20-0e_18-4 PE_20-1e_18-3	0.60	0.0011	1.37	0.0237
PE_19-0_18-2	0.43	0.0097	0.37	0.0458
PE_20-0e_18-1	1.04	0.9489	8.43	0.4088
PE_16-1_22-6 PE_18-2_20-5	0.54	<0.001	1.16	0.3278
PE_18-2_20-4 PE_18-1_20-5	0.63	0.0012	0.90	0.2515
PE_16-0_22-6 PE_16-1_22-5 PE_20-2_18-4	0.63	0.0012	0.90	0.2515
PE_18-1_20-4	1.13	0.7610	0.79	0.5920
PE_18-0_20-5	2.42	0.0006	0.25	<0.001
PE_16-0_22-4	0.28	0.0274	2.73	<0.001
PE_18-1_20-3 PE_18-2_20-2	1.75	<0.001	0.43	<0.001
PE_18-0_20-4	0.43	<0.001	2.20	<0.001
PE_20-1_18-2	0.47	0.0162	2.49	0.0021
PE_18-0_20-3	0.81	0.4037	1.05	0.6234
PE_18-0p_22-6 PE_18-1p_22-5	0.66	0.2525	0.87	0.2880
PE_17-0_22-6	0.23	<0.001	1.68	0.0377
PE_18-0p_22-5 PE_18-1p_22-4	0.71	0.0064	0.63	0.0118
PE_19-0_20-4	0.37	0.0116	0.91	0.7922
PE_20-0e_20-4 PE_18-0e_22-4	0.23	0.0243	4.31	0.0230
PE_18-1_22-6	0.37	0.0017	1.39	0.0188
PE_18-1_22-5	0.65	0.0104	0.69	0.0964
PE_18-0_22-6	0.44	0.0025	1.44	0.0038
PE_18-0_22-5	0.55	0.0230	2.14	<0.001
PE_18-0_22-4 PE_20-0_20-4	0.52	0.0114	7.32	0.0078
FA_12-0_Lauric acid	1.63	0.0057	0.71	0.0636
FA_14-1 (n-5)_Myristoleic acid	0.96	0.8466	0.95	0.7643
FA_14-0_Myristic acid	1.34	0.2913	0.79	0.3721
FA_15-0_Pentadecylic acid	0.77	0.3257	0.62	0.0766
FA_16-1 (n-7)_Palmitoleic acid	1.95	0.0563	0.46	0.0407
FA_16-0_Palmitic acid	1.33	0.0538	0.52	0.0011
FA_17-1 (n-7)_cis-10-Heptadecanoic acid	0.92	0.6288	0.75	0.0064
FA_17-0_Margaric acid	0.87	0.3921	0.86	0.3703
FA_18-4 (n-3)_Stearidonic acid	2.09	0.0066	0.34	0.0025
FA_18-3 (n-3)_Alpha-linolenic acid_(n-6)_gamma-Linolenic acid	2.06	0.0110	0.56	0.0212
FA_18-2 (n-6)_Linoleic acid	0.99	0.9353	0.62	0.0161
FA_18-1 (n-9)c_Oleic acid_(n-7)c_Vaccenic acid	0.94	0.6683	0.82	0.1300
FA_18-1 (n-9)t_Elaidic acid_(n-7)t_Vaccenic acid	0.89	0.4124	0.84	0.1767
FA_18-0_Stearic acid	1.61	0.0630	1.07	0.7040
FA_20-5 (n-3)_Eicosapentaenoic acid	3.15	0.0067	0.26	0.0053
FA_20-4 (n-6)_Arachidonic acid	1.02	0.8982	2.23	<0.001
FA_20-3 (n-6)_Dihomo-gamma-linolenic acid_(n-9)_Mead acid	1.87	0.0079	1.42	0.0215
FA_C20-2 (n-6)_cis-11-14-Eicosadienoic acid	0.88	0.4416	2.26	0.0011
FA_20-1 (n-9)_cis-11-Eicosenoic acid	0.34	0.0032	0.76	0.1514
FA_20-0_Arachidic acid	0.74	0.2275	1.06	0.6796
FA_21-0_Heneicosanoic acid	0.76	0.4236	1.25	0.4540
FA_22-6 (n-3)_Docosahexaenoic acid	1.19	0.1598	0.83	0.0938
FA_22-5 (n-6)_Docosapentaenoic acid	1.45	0.1591	0.87	0.5247

FA_22-4 (n-6)_Docosatetraenoic acid	0.63	0.0028	5.33	<0.001
FA_22-1 (n-9)_Erucic acid	0.75	0.2473	1.19	0.7362
FA_22-0_Behenic acid	0.87	0.3591	0.96	0.8010
FA_23-0_Tricosanoic acid	1.00	0.9965	0.85	0.3145
FA_24-1 (n-9)_Nervonic acid	0.33	0.0028	1.02	0.9197
AC_2-0	0.79	0.1063	0.35	<0.001
AC_14-0	0.43	0.0458	0.86	0.3675
AC_14-1	0.55	0.0595	0.70	0.1166
AC_16-0	0.73	0.0618	1.14	0.4618
AC_16-1	0.48	0.0366	0.98	0.8886
AC_18-0	0.72	0.0646	2.45	<0.001
AC_18-1	0.40	0.0055	1.68	0.0126
AC_18-2	0.39	0.0148	1.08	0.7192