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Lepr$^{db/db}$ Mice with Senescence Marker Protein-30 Knockout (Lepr$^{db/db}$Smp30$^{Y/−}$) Exhibit Increases in Small Dense-LDL and Severe Fatty Liver Despite Being Fed a Standard Diet

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Abstract

Background/Aims: The senescence marker protein-30 (SMP30) is a 34 kDa protein originally identified in rat liver that shows decreased levels with age. Several functional studies using SMP30 knockout (Smp30$^{Y/−}$) mice established that SMP30 functions as an antioxidant and protects against apoptosis. To address the potential role of SMP30 in nonalcoholic fatty liver disease (NAFLD) pathogenesis, we established Smp30$^{Y/−}$ mice on a Lepr$^{db/db}$ background (Lepr$^{db/db}$Smp30$^{Y/−}$ mice).

Research Design/Principal Findings: Male Lepr$^{db/db}$Smp30$^{Y/−}$ mice were fed a standard diet (340 kcal/100 g, fat 5.6%) for 16 weeks whereupon the lipid/lipoprotein profiles, hepatic expression of genes related to lipid metabolism and endoplasmic reticulum stress markers were analyzed by HPLC, quantitative RT-PCR and western blotting, respectively. Changes in the liver at a histological level were also investigated. The amount of SMP30 mRNA and protein in livers was decreased in Lepr$^{db/db}$Smp30$^{Y/+}$ mice compared with Lepr$^{db/+}$Smp30$^{Y/+}$ mice. Compared with Lepr$^{db/db}$Smp30$^{Y/+}$ mice, 24 week old Lepr$^{db/db}$Smp30$^{Y/−}$ mice showed: i) increased small dense LDL-cho and decreased HDL-cho levels; ii) fatty liver accompanied by numerous inflammatory cells and increased oxidative stress; iii) decreased mRNA expression of genes involved in fatty acid oxidation (PPARα) and lipoprotein uptake (LDLR and VLDLR) but increased CD36 levels; and iv) increased endoplasmic reticulum stress.

Conclusion: Our data strongly suggest that SMP30 is closely associated with NAFLD pathogenesis, and might be a possible therapeutic target for NAFLD.

Introduction

Metabolic syndrome has been described as the association of insulin resistance, hypertension, hyperlipidemia and obesity. Its prevalence has increased dramatically, mainly in developed countries. The hepatic manifestations of metabolic syndrome include nonalcoholic fatty liver disease (NAFLD) and its progressive variant, nonalcoholic steatohepatitis (NASH) [1,2]. Several animal models have been proposed for NAFLD and NASH research [3]. Since leptin plays a major role in food intake and energy expenditure, total leptin deficiency or leptin resistance can lead to massive obesity, type 2 diabetes, dyslipidemia and fatty liver. Therefore, many investigations pertaining to NAFLD/NASH have been carried out in genetic leptin-deficient ob/ob mice or leptin-resistant db/db mice that were fed a high fat diet (HFD) or the methionine/choline deficiency diet [3–5]. However, these models differ significantly from the human NAFLD/NASH phenotype in a number of pathogenically important ways.

The senescence marker protein-30 (SMP30) is a 34 kDa protein that was originally identified in rat liver and its levels decrease with age [6]. We previously reported that SMP30 participates in Ca^{2+} efflux by activating the calmodulin-dependent Ca^{2+}-pump that confers resistance to cell injury caused by high intracellular Ca^{2+} concentrations [7]. We identified SMP30 as a gluconolactonase

(GNL) that is involved in L-ascorbic acid biosynthesis in mammals, and have established SMP30-knockout (KO) mice [8]. The livers of SMP30-KO mice are highly susceptible to tumor necrosis factor-α (TNF-a) and Fas-mediated apoptosis, indicating that SMP30 has an anti-apoptotic effect [9]. SMP30-KO mice showed mitochondrial damage and abnormal accumulation of triglycerides, cholesterol, and phospholipids in the liver [10]. In addition, we reported that decreased SMP30 levels contribute to lowered glucose tolerance [11]. These results are in agreement with several functional studies, which also established that SMP30 functions as an antioxidant and anti-apoptotic protein [12–15].

To address the potential role of SMP30 in NAFLD/NASH pathogenesis, we generated SMP30-KO mice on a $Lepr^{db/db}$ background ($Lepr^{db/db}Smp30^{Y/-}$) and investigated the lipid/lipoprotein profiles, hepatic expression of genes relevant to lipid metabolism and histological changes in the livers of $Lepr^{db/db}Smp30^{Y/-}$ mice fed a standard diet. Here we show that despite being fed a standard diet, $Lepr^{db/db}Smp30^{Y/-}$ mice have altered lipoprotein components and severe fatty liver accompanied by increased inflammation and oxidative stress induced by mitochondrial and endoplasmic reticulum dysfunction.

Materials and Methods

Animal crossing and genotyping, and experimental protocol

We used type 2 diabetic obese $Lepr^{db/db}$ mice with a C57BLKS/J background. Male $Lepr^{db/+}$ mice were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). SMP30-knockout (KO) mice with a C57BL/6 background were established and maintained as described previously [8,9]. Heterozygous SMP30-KO male mice do not exist, because the $Smp30$ gene is located on the X chromosome. SMP30-KO mice cannot synthesize vitamin C in $vivo$, because in mammals SMP30 is the penultimate enzyme in the vitamin C biosynthetic pathway [8]. To maintain vitamin C levels in tissues that were similar to that of wild type mice, and to eliminate any possible confounding influences of vitamin C deficiency, these mice were given free access to water supplemented with 1.5 g/L vitamin C and 10 μM ethylenediaminetetraacetic acid (EDTA) to avoid the effects of vitamin C deficiency [16]. As schematically illustrated in Figure 1A, male $Lepr^{db/+}$ mice were first crossed with female $Smp30^{-/-}$ mice to produce male $Lepr^{db/+}Smp30^{Y/-}$ mice and female $Lepr^{db/+}Smp30^{+/-}$ mice. The SMP30 mutant mice genotypes were determined as described previously [9]. Next, male $Lepr^{db/+}Smp30^{Y/-}$ and female $Lepr^{db/+}Smp30^{+/-}$ mice were interbred to produce homozygous $Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice and heterozygote control $Lepr^{db/+}Smp30^{Y/-}$ and $Lepr^{db/+}Smp30^{Y/+}$ mice. The mutant $Lepr^{db}$ gene was identified by restriction enzyme digestion of PCR products. In brief, $Lepr^{db}$ gene PCR products were amplified by PCR using genomic DNA and forward (5′-AGAACGGA-CACTCTTTGAAGTCTC-3′) and reverse (5′-CATTCAAAC-CATAGTTTAGGTTTGTGT-3′) primers. PCR products were then digested by AfaI (Takara Bio Inc., Shiga, Japan) and analyzed by agarose gel electrophoresis. The mutant $Lepr^{db}$ gene showed two bands of 108 bp and 27 bp while the wild type allele showed one 135 bp band.

We prepared four groups of five eight week old male mice, with each group having four genotypes: $Lepr^{db/+}Smp30^{Y/+}$, $Lepr^{db/+}Smp30^{Y/-}$, $Lepr^{db/db}Smp30^{Y/+}$, and $Lepr^{db/db}Smp30^{Y/-}$. All mice were fed a vitamin C free-standard diet (CL-2; 340 kcal/100 g, fat 5.6%, CLEA Japan, Tokyo, Japan) for 16 weeks. $Lepr^{db/+}Smp30^{Y/-}$ mice and $Lepr^{db/db}Smp30^{Y/-}$ mice had free access to 1.5 g/L vitamin C water containing 10 μM EDTA, whereas $Lepr^{db/+}$

$Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice had 10 μM EDTA water. Mice were maintained on a 12 h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology (Permit Number: 12016).

Blood and liver tissue collection

All mice were fasted for 16 h and anesthetized at the age of 24 weeks. Blood was obtained from the inferior vena cava, anticoagulated with EDTA, and subsequently centrifuged at 880×g for 15 min at 4°C. Mice were systemically perfused with ice-cold phosphate buffered saline through the hepatic portal vein to wash out remaining blood cells and then the livers were removed. The whole body subcutaneous fat was collected and the weight measured. The livers were immersed in RNA$later^®$ (Life Technologies Corp., Carlsbad, CA, USA) for RNA extraction and fixed with 10% neutral buffered formalin for histological analysis or frozen in liquid nitrogen for biochemical analysis. All samples were stored at −80°C until use.

Biochemical analysis of blood

Blood glucose levels were measured using a glucometer (Glutest Every; Sanwa Kagaku Kenkyusho Co., Ltd., Aichi, Japan). Plasma insulin levels were measured using an enzyme-linked immunoassay system (Ultra sensitive mouse insulin ELISA kit; Morinaga Institute of Biological Science Inc., Kanagawa, Japan). Plasma total cholesterol (T-cho), triglyceride (TG), phospholipid (PL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured by enzymatic assay kits (Wako Pure Chemicals Industries, Osaka, Japan).

Western blotting analysis

Livers were homogenized in ice-cold homogenization buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM phenyl methanesulfonyl fluoride, and a protease inhibitor cocktail (cOmplete, EDTA-free; Roche Diagnostics GmbH, Mannheim, Germany)) for 30 seconds using a high speed homogenizer (POLYTRON® PT-MR 2100; Kinematica AG, Switzerland). The homogenate was then centrifuged at 21,000×g for 10 min at 4°C. The supernatants were boiled for 5 min with a lysis buffer containing 0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue at a ratio of 1:1. Total protein equivalents for each sample were separated on a 14% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with the primary antibody, followed by incubation with a horseradish peroxidase-linked goat anti-rabbit IgG (Bio-Rad Laboratories, Tokyo, Japan). The primary antibodies used were: anti-rat SMP30 rabbit polyclonal antibody (Cosmo Bio Co., Ltd., Tokyo, Japan), anti-phosphorylated eukaryotic initiation factor-2α (p-eIF2α) rabbit monoclonal antibody (#3597, Cell Signaling Technology, Beverly, MA) and anti-C/EBP homologous protein (CHOP) rabbit polyclonal antibody (sc-575, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Cell Signaling Technology, Beverly, MA) as a loading control. After washing, immunoreactivity was detected using ECL chemiluminescence reagents (ECL plus, GE Healthcare Japan, Tokyo, Japan). Chemiluminescence signals were quantified with a LAS-3000 imaging system (Fujifilm, Tokyo, Japan). The mean signals from five $Lepr^{db/+}Smp30^{Y/+}$ mice were assigned a relative value of 1.0. The total protein concentration was determined with a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA) using bovine serum albumin as a standard.
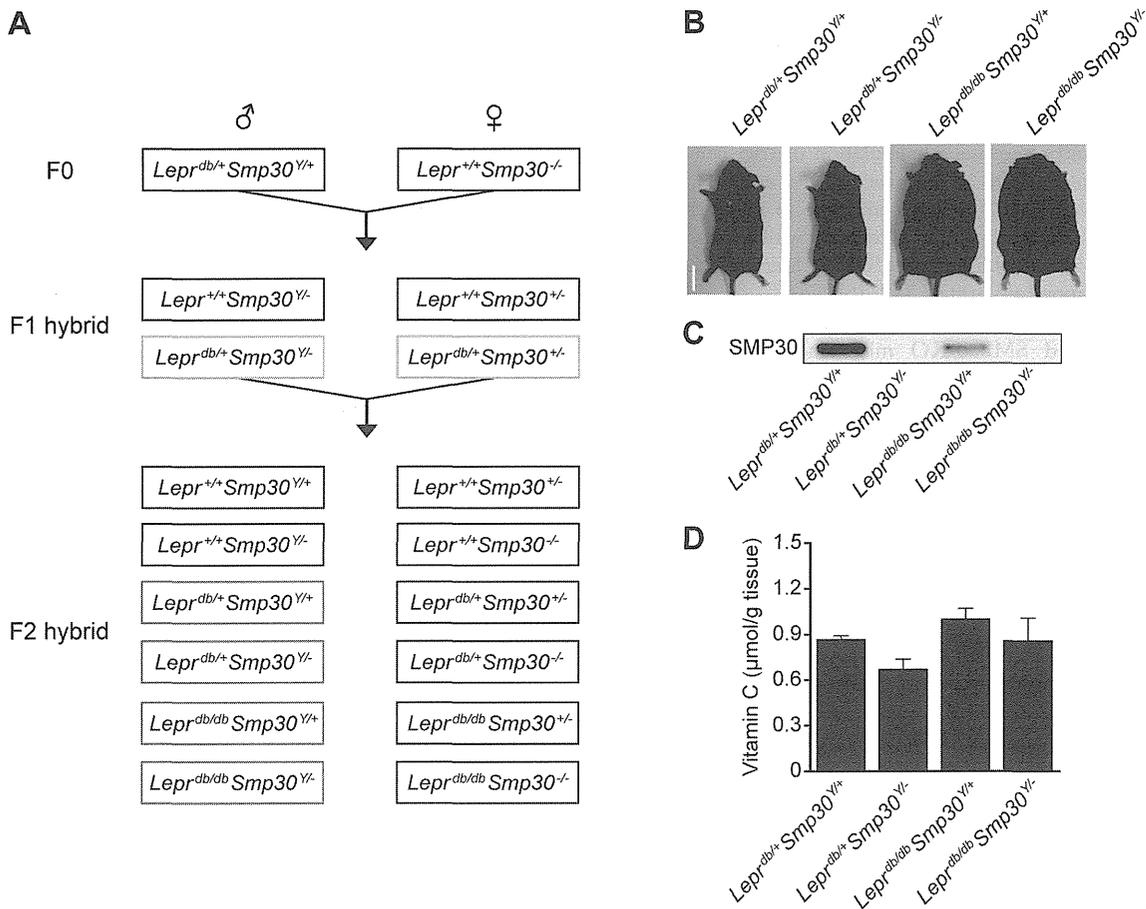
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	♂	♀
F0	$Lepr^{db/+}Smp30^{Y/+}$	$Lepr^{+/+}Smp30^{-/-}$

F1 hybrid

$Lepr^{+/+}Smp30^{Y/-}$	$Lepr^{+/+}Smp30^{+/-}$
$Lepr^{db/+}Smp30^{Y/-}$	$Lepr^{db/+}Smp30^{+/-}$

F2 hybrid

$Lepr^{+/+}Smp30^{Y/+}$	$Lepr^{+/+}Smp30^{+/-}$
$Lepr^{+/+}Smp30^{Y/-}$	$Lepr^{+/+}Smp30^{-/-}$
$Lepr^{db/+}Smp30^{Y/+}$	$Lepr^{db/+}Smp30^{+/-}$
$Lepr^{db/+}Smp30^{Y/-}$	$Lepr^{db/+}Smp30^{-/-}$
$Lepr^{db/db}Smp30^{Y/+}$	$Lepr^{db/db}Smp30^{+/-}$
$Lepr^{db/db}Smp30^{Y/-}$	$Lepr^{db/db}Smp30^{-/-}$

Figure 1. Establishment of $Lepr^{db/db}Smp30^{Y/-}$ mice. (A) Generation of $Lepr^{db/db}Smp30^{Y/-}$ mice. F1 hybrid mice (in green boxes) were crossed to produce the experimental $Lepr^{db/+}Smp30^{Y/+}$, $Lepr^{db/+}Smp30^{Y/-}$, $Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice (in red boxes). (B) Appearance of $Lepr^{db/db}Smp30^{Y/-}$ mice at 24 weeks of age. (C) Western blot analysis of SMP30 protein levels in livers from each experimental group at 24 weeks of age. (D) Vitamin C concentration in livers from each experimental group of mice at 24 weeks of age. Values are given as means ± SEM of five animals.
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Measurement of vitamin C levels in the liver

Livers were homogenized in 14 vol 5.4% metaphosphate and 1 mM EDTA, and the homogenate was then centrifuged at $21,000 \times g$ for 15 min at 4°C. Vitamin C was analyzed by high-performance liquid chromatography (HPLC) using an Atlantis dC18 5 μm column (4.6×150 mm; Nihon Waters, Tokyo, Japan) [17]. The mobile phase was 50 mM phosphate buffer (pH 2.8), 0.2 g/L EDTA, and 2% methanol at a flow rate of 1.3 ml/min, and electrical signals were recorded using an electrochemical detector equipped with a glassy carbon electrode at +0.6 V.

Measurement of T-cho, TG and PL in the liver

Liver tissues were homogenized with 2 vol. of water using a handy homogenizer (Moji-mojikun; Nippon Genetics, Tokyo, Japan). Homogenates were added to a chloroform-methanol (2:1; v/v) mixture, and centrifuged at $21,000 \times g$ for 10 min at 4°C. The supernatant organic phase was then collected, dried under nitrogen gas and resolubilized in 2-propanol. T-cho, TG and PL concentrations in total lipid extracts were determined using commercial enzymatic kits (Wako Pure Chemical Industries, Osaka, Japan).

Thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was estimated by the amounts of TBARS in the liver that were determined according to the method of Ohkawa et al. [18]. The livers were first homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenates were then centrifuged at $15,000 \times g$ for 30 min at 4°C and the supernatant was used for further assays. One volume of sample was mixed thoroughly with two volumes of stock solution (15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 mM HCl). The mixture was then heated for 30 min in a boiling water bath. After cooling, the flocculate precipitate was removed by centrifugation at $1,000 \times g$ for 10 min and the absorbance (OD 532 nm) of the sample was measured. The TBARS levels are expressed as the equivalent amounts of malondialdehyde produced from 1,1,3,3-tetramethoxypropane.

HPLC analysis of plasma lipoproteins

Plasma lipoproteins were analyzed using a HPLC service (LipoSEARCH) at Skylight Biotech Inc. (Akita, Japan), as previously described [19]. In brief, 10 μL of whole plasma was injected into two connected columns (300×7.8 mm) packed with TSKgel LipopropakXL (Tosoh, Tokyo, Japan) and lipoproteins were separated with 0.05 mol/L Tris-buffered acetate (pH 8.0) containing 0.3 mol/L sodium acetate, 0.05% sodium azide, and

0.005% Brij-35 at a flow rate of 0.7 mL/min. The column effluent was split equally into two lines by a P-460 MicroSplitter (Upchurch Scientific Inc., Oak Harbor, WA, USA); one effluent portion was mixed with cholesterol reagent (Determiner L TC; Kyowa Medex Co., Ltd., Tokyo, Japan) and the other with TG reagent (Determiner L TG; Kyowa Medex). We defined 5 VLDL subclasses (fraction no. 3–7, 30–80 nm), 6 LDL subclasses (fraction no. 8–13, 16–30 nm) and 7 HDL subclasses (fraction no. 14–20, 8–16 nm) using 20 component peaks categorized on the basis of lipoprotein particle size (diameter).

Histological and immunohistochemical examination of liver

To evaluate histological changes, fixed liver sections were subjected to hematoxylin-eosin (H&E) staining or immunohisto-chemical staining with an anti-4-hydroxy-2-nonenal (4-HNE) protein adducts monoclonal antibody (1:40 dilution; Nikken SEIL, Shizuoka, Japan).

The H&E-stained specimens were anonymized, and five different areas per mouse were randomly selected by a researcher. These specimens were scored by two independent investigators blinded to sample identity according to the NASH activity score (NAS) [20] for the degree of steatosis (0–3), lobular inflammation (0–3) and hepatocellular ballooning (0–2). The average of the two investigators' scores was regarded as the score for each mouse.

4-HNE was detected by indirect immunoperoxidase staining using corresponding Histofine Simple Stain MAX-PO kits (Nichirei Biosciences, Tokyo, Japan) and 3, 3-diaminobenzidine (DAB) as a chromogenic substrate. After DAB staining, nuclei were counterstained with Mayer's hematoxylin. Two independent observers evaluated the intensity of 4-HNE immunostaining and assigned scores of 0, 1, 2, or 3 (negative (including faint staining), weak, moderate, or strong, respectively).

RNA isolation, first-strand cDNA synthesis, and gene expression analysis

Liver tissue was finely ground with a liquid nitrogen-cooled mortar and pestle and homogenized in ice-cold TRIzol reagent (Life Technologies, Carlsbad, CA, USA) before isolation of total RNA according to the manufacturer's instructions. Total RNA (0.5 μg) was reverse-transcribed using PrimeScript RT Master Mix (TaKaRa Bio Inc., Shiga, Japan) for first-strand cDNA synthesis with an oligonucleotide dT primer and random hexamer priming according to the manufacturer's recommendations.

The mRNA expression levels of the following proteins: acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), sterol regulatory element-binding protein 1c (SREBP1c), SREBP2, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoAR), peroxisome proliferator-activated receptor-α (PPARα), medium-chain acyl-CoA dehydrogenase (MCAD), microsomal triglyceride transfer protein (MTP), apolipoprotein-B100 (ApoB100), LDL receptor (LDLR) and VLDL receptor (VLDLR), CD36 and spliced X-box binding protein 1 (sXBP1), all of which are involved in lipid and lipoprotein metabolism, were quantitated using real-time reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using a Thermal Cycler Dice Real Time System II (TaKaRa Bio Inc.) and real-time SYBR® Premix Ex Taq^{TM} (TaKaRa Bio Inc.) according to the manufacturer's instructions. The specific primers for the target genes and β-actin are described in Table 1. The following PCR conditions were used: 1 cycle for 30 s at 95°C, followed by 40 cycles for 5 s at 95°C, and 30 s at 60°C. The product specificity generated for each primer set was examined for each fragment using a melting curve and gel electrophoresis. The relative expression levels of each targeted gene were normalized to β-actin threshold cycle (CT) values and quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method as previously described [21]. Signals from $Lepr^{db/+}Smp30^{Y/+}$ mice were assigned a relative value of 1.0. Five mice from each group were examined, and real time RT-PCR was run in duplicate for each sample.

Statistical analysis

Data are expressed as means ± SEM. Statistical differences between groups were determined by one-way analysis of variance (ANOVA) with Scheffe's post hoc test. A P value<0.05 was considered to be statistically significant.

Results

Generation of $Lepr^{db/db}Smp30^{Y/-}$ mice

As shown in Fig. 1A and B, we established $Lepr^{db/db}Smp30^{Y/-}$ mice, which were born at the expected Mendelian ratios and by 24 weeks of age had appearances that were indistinguishable from obese $Lepr^{db/db}Smp30^{Y/+}$ mice. Western blot analysis of liver tissue from $Lepr^{db/+}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice demonstrated that these animals lacked SMP30 protein (Fig. 1C). There were no significant differences found in the liver vitamin C concentration among any of the experimental groups (Fig. 1D).

Given the difference in SMP30 protein levels observed for $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice (Fig. 1C), we next quantified the amounts of SMP30 mRNA and protein and found that they were decreased by 25% and 47%, respectively, in $Lepr^{db/db}Smp30^{Y/+}$ mice as compared to $Lepr^{db/+}Smp30^{Y/+}$ mice (both $P<0.01$) (Fig. 2).

$Lepr^{db/db}Smp30^{Y/-}$ mice had manifestations of metabolic syndrome

The physiological and blood biochemical parameters of 24 week old animals of the four groups are presented in Table 2. Compared with $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice, the food intake of $Lepr^{db/db}Smp30^{Y/-}$ mice during the experimental period was significantly increased by 33% and 40%, respectively ($P<0.001$). Meanwhile, there were no significant differences between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice ($P=0.07$). The body weight of $Lepr^{db/db}Smp30^{Y/-}$ mice was 79% and 87% higher than $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice, respectively ($P<0.001$). There were no significant differences in body weight between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice. Likewise, the epididymal and subcutaneous fat weight in $Lepr^{db/db}Smp30^{Y/-}$ mice were significantly higher than those of $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice ($P<0.001$), although no significant differences in epididymal or subcutaneous fat weight were observed between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice.

Fasting blood glucose concentrations were significantly higher in $Lepr^{db/db}Smp30^{Y/-}$ mice (239.8±26.3 mg/dL) than in $Lepr^{db/+}Smp30^{Y/+}$ (71.4±5.8 mg/dL, $P<0.001$), $Lepr^{db/+}Smp30^{Y/-}$ (82.2±4.3 mg/dL, $P<0.001$) and $Lepr^{db/db}Smp30^{Y/+}$ mice (162.0±15.2 mg/dL, $P<0.05$). Fasting plasma insulin concentrations in $Lepr^{db/db}Smp30^{Y/-}$ mice were also higher than in $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice ($P<0.001$). There were no significant differences in fasting plasma levels between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice.

Although there was a non-significant increase in plasma TG levels in the two groups of $Lepr^{db/db}$ mice ($Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$) compared with the two groups of $Lepr^{db/+}$ mice ($Lepr^{db/+}Smp30^{Y/-}$ and $Lepr^{db/+}Smp30^{Y/+}$), there were no significant

Table 1. Primer sequences for use in real-time quantitative RT-PCR.

Gene name		Sequence	Size (bp)	GenBank accession no.
Acetyl-CoA carboxylase	Forward	5'-AGCGACATGAACACCGTACTGAA-3'	106	NM133360
	Reverse	5'-TAGGGTCCCGGCCACATAAC-3'		
Fatty acid synthase	Forward	5'-ATTGGCTCCACCAAATCCAAC-3'	90	NM007988
	Reverse	5'-CCCATGCTCCAGGGATAACAG-3'		
Sterol regulatory element-binding protein-1c	Forward	5'-AGCCTGGCCATCTGTGAGAA-3'	132	NM011480
	Reverse	5'-CAGACTGGTACGGGCCACAA-3'		
Sterol regulatory element-binding protein-2	Forward	5'-CCCACTCAGAACACCAAGCAT-3	67	NM033218
	Reverse	5'-TGGCAGTAGCTCGCTCTCGT-3'		
HMGCoA reductase	Forward	5'- TGTCCTTGATGGCAGCCTTG-3'	183	NM 008255
	Reverse	5'- CCGCGCTTCAGTTCAGTGTC-3'		
Peroxisome proliferator-activated receptor-α	Forward	5'-CTCAGGGTACCACTACGGAGTTCAC-3'	111	NM001113418
	Reverse	5'-TGAATCTTGCAGCTCCGATCAC-3'		
Medium-chain acyl-CoA dehydrogenase	Forward	5'- TGAGTGGCGGCCATTAAGA-3'	179	NM007382
	Reverse	5'- CGGCTTCCACAATGAATCCAG-3'		
Microsomal triglyceride transfer protein	Forward	5'-CATCTCCACAGTGCAGTTCTCACA-3'	167	NM001163457
	Reverse	5'-GGAGTTCACATCCGGCCACTA-3'		
Apolipoprotein-B100	Forward	5'-CAGGTGGCCACAGCCAATAA-3'	161	NM009693
	Reverse	5'-ACTGCAGGTCTGGCTCAGGA-3'		
LDL receptor	Forward	5'- CTAGCAAGTGGGTGTGCGATG-3'	100	NM010700
	Reverse	5'- CTGAATTGATTGGACTGACAGGTGA -3'		
VLDL receptor	Forward	5'- TGGCGTGTGCAAGGCAGTA-3'	145	NM013703
	Reverse	5'- TGCAATGTCCGCATCGAGA-3'		
CD36	Forward	5'-CCCAGATGACGTGGCAAAGA-3'	172	NM001159555
	Reverse	5'-TCCAACAGACAGTGAAGGCTCAA-3'		
Spliced XBP1	Forward	5'-GCTGAGTCCGCAGCAGGT-3'	86	NM013842
	Reverse	5'-GAATCTGAAGAGGCAACAGTGTCA-3'		
Senescence marker protein-30	Forward	5'-GAGGCAGCCTGATGCTGGTAA-3'	92	NM009060
	Reverse	5'-GAGCTGCAGTTCACCCTGCATA-3'		
β-actin	Forward	5'-CATCCGTAAAGACCTCTATGCCAAC-3'	171	NM007393
	Reverse	5'-ATGGAGCCACCGATCCACA-3'		

differences in plasma TG levels among any of the experimental groups. Both plasma T-cho and PL concentrations in $Lepr^{db/db}Smp30^{Y/-}$ mice were significantly higher than those in $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice (both $P<0.001$). There were no significant differences in plasma T-cho and PL concentrations between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice.

Plasma AST and ALT concentrations in $Lepr^{db/db}Smp30^{Y/-}$ mice (136.8±27.3 and 111.0±26.1 IU/L, respectively) were significantly higher than those of $Lepr^{db/+}Smp30^{Y/+}$ (32.8±4.6 IU/L, $P<0.01$ and 11.2±15 IU/L, $P<0.01$, respectively) and $Lepr^{db/+}Smp30^{Y/-}$ (37.6±7.1 IU/L, $P<0.01$ and 28.8±14.0 IU/L, $P<0.01$, respectively) mice. There were no significant differences in plasma AST and ALT concentrations between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice.

$Lepr^{db/db}Smp30^{Y/-}$ mice show decreased HDL-cho and increased small dense LDL-cho levels and increased LDL-cho/HDL-cho ratios

HPLC analysis of the plasma lipoprotein profile with cholesterol reagents is shown in Fig. 3A. Either with or without $Smp30$, the levels of VLDL- (Fr. 3–7), LDL- (Fr. 8–11), very-small particle size LDL- (Fr. 12–13) and HDL-cho (Fr.14–20) as well as the LDL-cho/HLD-cho ratio were significantly higher in $Lepr^{db/db}$ mice ($Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice) compared to $Lepr^{db/+}$ mice ($Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice) ($P<0.001$) (Fig. 3B–F). There were no significant differences in VLDL-cho levels in $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice (Fig. 3B). Although there were no significant differences in LDL-cho (Fr. 8–11) levels in $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice (Fig. 3C), the very-small sized LDL-cho particles of Fr.12 and Fr.13 that correspond to small dense LDL-cho (sdLDL-cho) in
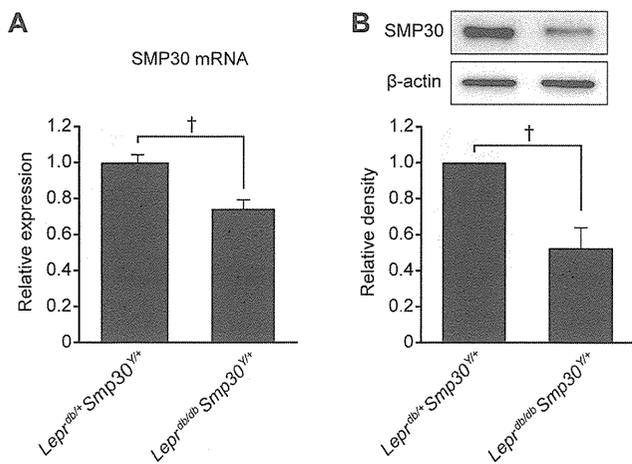
A

SMP30 mRNA

B

SMP30
β-actin

Figure 2. Decrease in hepatic SMP30 levels in $Lepr^{db/db}$ mice. (A) SMP30 mRNA and (B) protein levels in livers from $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice. Values are given as means ± SEM of five animals. $^{†}P<0.01$.
doi:10.1371/journal.pone.0065698.g002

$Lepr^{db/db}Smp30^{Y/-}$ mice (78.6±3.2 mg/dL) were significantly higher than in $Lepr^{db/db}Smp30^{Y/+}$ mice (66.3±3.5 mg/dL, $P<0.05$) (Fig. 3D), while HDL-cho levels in $Lepr^{db/db}Smp30^{Y/-}$ mice (173.0±6.1 mg/dL) were significantly lower than that of $Lepr^{db/db}Smp30^{Y/+}$ mice (221.0±4.8 mg/dL, $P<0.001$) (Fig. 3E). The LDL-cho/HDL-cho ratio in $Lepr^{db/db}Smp30^{Y/-}$ mice was significantly higher than that in $Lepr^{db/db}Smp30^{Y/+}$ mice ($P<0.001$) (Fig. 3F). Likewise, the LDL-cho/HDL-cho ratio in $Lepr^{db/+}Smp30^{Y/-}$ mice was significantly higher than that of $Lepr^{db/+}Smp30^{Y/+}$ mice ($P<0.01$) (Fig. 3F).

Increased hepatic lipid (TG, T-cho and PL) and lipid peroxidation (TBARS) levels in $Lepr^{db/db}Smp30^{Y/-}$ mice

As shown in Fig. 4A–D, hepatic TG, T-cho, PL and TBARS concentrations were significantly higher in $Lepr^{db/db}Smp30^{Y/-}$ mice than $Lepr^{db/+}Smp30^{Y/+}$ or $Lepr^{db/+}Smp30^{Y/-}$ mice ($P<0.001$). Compared with $Lepr^{db/db}Smp30^{Y/+}$ mice, hepatic TG, T-cho and PL concentrations were lower and TBARS concentrations were higher in $Lepr^{db/db}Smp30^{Y/-}$ mice.

$Lepr^{db/db}Smp30^{Y/-}$ mice show fatty liver accompanied by inflammatory cells and oxidative stress despite being fed a standard diet

As shown in Fig. 5A, hepatic histological examination revealed increased steatosis in $Lepr^{db/db}Smp30^{Y/+}$ mice and increased steatosis accompanied by inflammatory cells in $Lepr^{db/db}Smp30^{Y/-}$ mice. In contrast, no histological abnormalities were observed for both $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ animals. Fibrosis was not observed in any of the groups.

Regarding the NASH activity score, 24 week old $Lepr^{db/db}Smp30^{Y/-}$ mice had a total score of 4.4±0.2, which was significantly higher than that of $Lepr^{db/db}Smp30^{Y/+}$ mice (3.2±0.4, $P<0.05$). In immunohistochemical examinations using an anti-4HNE antibody, the 4-HNE intensity scores were significantly higher in $Lepr^{db/db}Smp30^{Y/-}$ mice (1.8±0.2) than in $Lepr^{db/db}Smp30^{Y/+}$ (1.2±0.2, $P<0.05$), $Lepr^{db/+}Smp30^{Y/-}$ (0.8±0.2, $P<0.01$) and $Lepr^{db/+}Smp30^{Y/+}$ (0.2±0.2, $P<0.001$) (Fig. 5B) mice.

Altered hepatic expression of lipid/lipoprotein metabolism-related genes in $Lepr^{db/db}Smp30^{Y/-}$ mice

Next, we investigated the hepatic expression of genes related to lipid/lipoprotein metabolism in $Lepr^{db/db}Smp30^{Y/-}$ mice (Fig. 6). Regarding lipogenesis genes, hepatic expression of ACC, FAS and SREBP1c were markedly higher in $Lepr^{db/db}$ mice ($Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$) than in $Lepr^{db/+}$ mice ($Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$) with or without the SMP30 gene. SREBP1c mRNA levels in $Lepr^{db/db}Smp30^{Y/-}$ mice were significantly lower

Table 2. Physiological, blood and biochemical parameters in four experimetal groups of mice.

	$Lepr^{db/+}Smp30^{Y/+}$	$Lepr^{db/+}Smp30^{Y/-}$	$Lepr^{db/db}Smp30^{Y/+}$	$Lepr^{db/db}Smp30^{Y/-}$
Food intake (g/day/mouse)	4.2±0.1	3.9±0.1	5.0±0.2§,¶	5.5±0.1§,¶
Body weight (g)	34.4±1.7	32.9±0.9	69.4±2.2§,¶	61.5±3.0§,¶
Epididymal fat weight (g)	1.12±0.18	1.10±0.06	2.60±0.13§,¶	2.42±0.18§,¶
Subcutaneous fat weight (g)	1.20±0.36	1.40±0.09	12.40±0.80§,¶	10.82±0.66§,¶
Fasting blood glucose (mg/dL)	71.4±5.8	82.2±4.3	162.0±15.2$^{*,#}$	239.8±26.3§,¶,**
Fasting plasma insulin (ng/mL)	1.0±0.2	1.3±0.5	11.6±1.8§,¶	9.2±0.8§,¶
Plasma triglyceride (mg/dL)	33.4±3.7	34.2±3.5	66.6±16.9	78.8±15.6
Plasma cholesterol (mg/dL)	59.6±4.0	58.4±1.7	358.8±58.8§,¶	333.2±68.1§,¶
Plasma phospholipid (mg/dL)	110.8±11.0	125.6±5.2	537.2±44.4§,¶	522.2±53.8§,¶
Plasma aspartate aminotransferase (IU/L)	32.8±4.6	37.6±7.1	98.3±3.7	136.8±27.3†,‡
Plasma alanine aminotransferase (IU/L)	11.2±1.5	28.8±14.0	86.3±7.6*	111.0±26.1$^{†,#}$

$^{*}P<0.05$,
$^{†}P<0.01$ and
$^{§}P<0.001$ versus $Lepr^{db/+}Smp30^{Y/+}$.
$^{#}P<0.05$,
$^{‡}P<0.01$ and
$^{¶}P<0.001$ versus $Lepr^{db/+}Smp30^{Y/-}$.
$^{**}P<0.05$ versus $Lepr^{db/db}Smp30^{Y/+}$.
Values are given as means ± SEM of five animals.
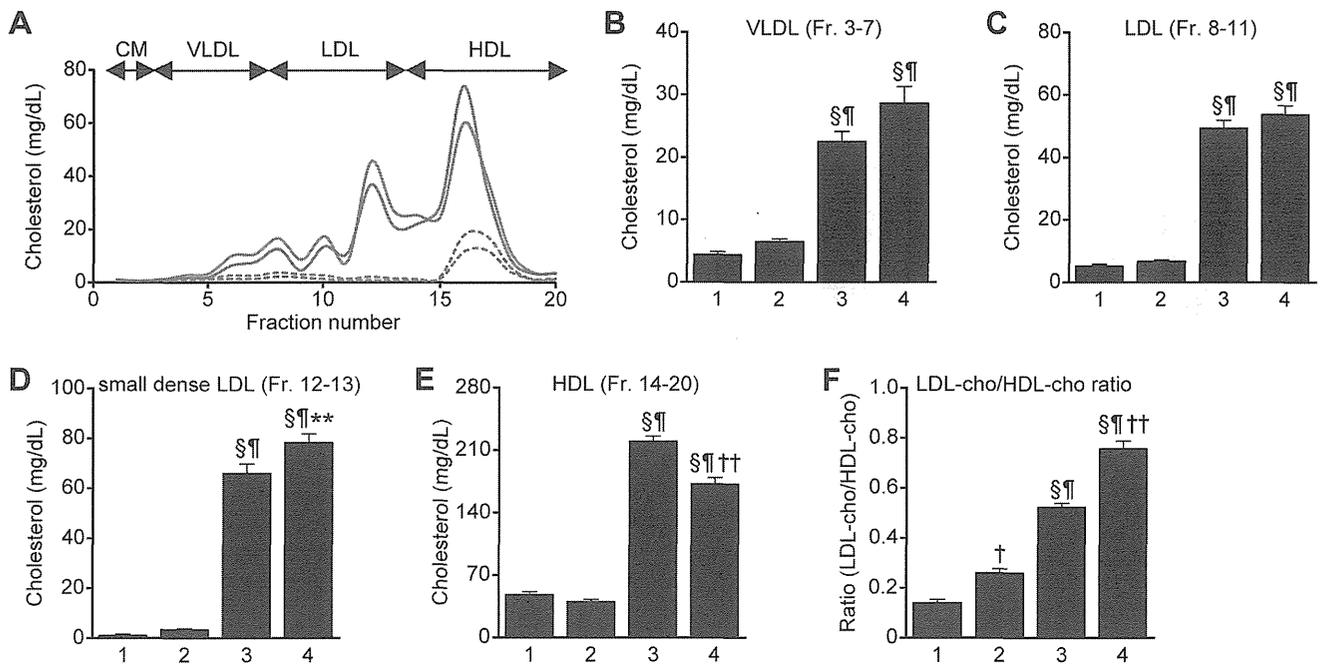doi:10.1371/journal.pone.0065698.t002

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Figure 3. Increase in small dense LDL-cho and decrease in HDL-cho in plasma from $Lepr^{db/db}Smp30^{Y/-}$ mice. (A) HPLC lipoprotein profile with cholesterol reagent and (B–F) cholesterol content in four subfractions according particle size, VLDL; Fr. 3–7, LDL; Fr. 8–11, small dense LDL; Fr. 12–13, HDL; Fr. 14–20 and LDL/HDL ratio in the four experimental groups. Blue dashed line and lane 1, $Lepr^{db/+}Smp30^{Y/+}$ mice; Red dashed line and lane 2, $Lepr^{db/+}Smp30^{Y/-}$ mice; Blue line and lane 3, $Lepr^{db/db}Smp30^{Y/+}$ mice; Red line and lane 4; $Lepr^{db/db}Smp30^{Y/-}$ mice. Values are given as means ± SEM of five animals. $^{†}P<0.01$ and $^{§}P<0.001$ *versus* $Lepr^{db/+}Smp30^{Y/+}$, $^{¶}P<0.001$ *versus* $Lepr^{db/+}Smp30^{Y/-}$, $^{**}P<0.05$ and $^{††}P<0.001$ *versus* $Lepr^{db/db}Smp30^{Y/+}$.
doi:10.1371/journal.pone.0065698.g003

than that in $Lepr^{db/db}Smp30^{Y/+}$ mice ($P<0.05$), although there was no significant difference in *ACC* and *FAS* mRNA expression
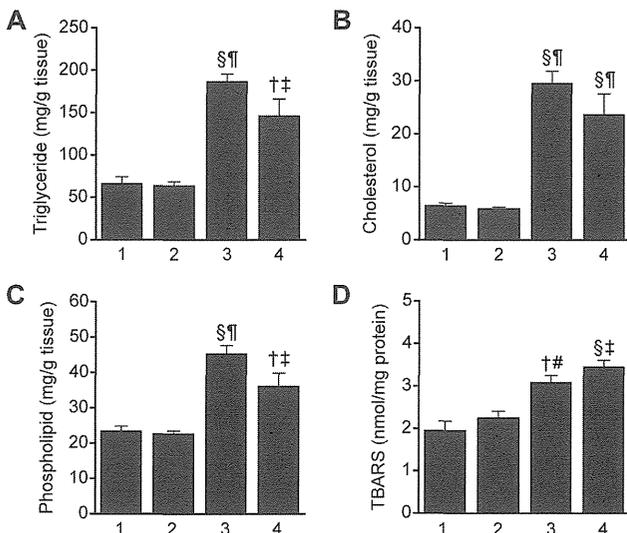

Figure 4. Increase in TBARS in livers from $Lepr^{db/db}Smp30^{Y/-}$ mice. (A) Triglyceride, (B) Cholesterol, (C) Phospholipid and (D) TBARS content in livers from the four experimental groups. Lane 1, $Lepr^{db/+}Smp30^{Y/+}$ mice. Lane 2, $Lepr^{db/+}Smp30^{Y/-}$ mice. Lane 3, $Lepr^{db/db}Smp30^{Y/+}$ mice. Lane 4, $Lepr^{db/db}Smp30^{Y/-}$ mice. $^{†}P<0.01$ and $^{§}P<0.001$ *versus* $Lepr^{db/+}Smp30^{Y/+}$, $^{¶}P<0.001$ *versus* $Lepr^{db/+}Smp30^{Y/-}$, $^{**}P<0.05$ and $^{††}P<0.001$ *versus* $Lepr^{db/db}Smp30^{Y/+}$. Values are given as means ± SEM of five animals.
doi:10.1371/journal.pone.0065698.g004

between $Lepr^{db/+}Smp30^{Y/+}$ mice and $Lepr^{db/+}Smp30^{Y/-}$ mice or between $Lepr^{db/db}Smp30^{Y/+}$ mice and $Lepr^{db/db}Smp30^{Y/-}$ mice. On the other hand, independent of the *SMP30* gene, *SREBP2* and *HMGCoAR* mRNA levels were significantly lower in $Lepr^{db/db}$ mice than those in $Lepr^{db/+}$. There were no significant differences in *SREBP2* and *HMGCoAR* expression levels between $Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice or between $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice.

mRNA levels of *PPARα*, which is involved in fatty acid catabolism, were significantly lower in $Lepr^{db/db}Smp30^{Y/-}$ mice than in $Lepr^{db/+}Smp30^{Y/+}$ ($P<0.001$), $Lepr^{db/+}Smp30^{Y/-}$ ($P<0.001$) or $Lepr^{db/db}Smp30^{Y/+}$ ($P<0.05$) mice. Importantly, *PPARα* mRNA levels were significantly lower in $Lepr^{db/+}Smp30^{Y/-}$ mice compared to $Lepr^{db/+}Smp30^{Y/+}$ mice ($P<0.05$). No significant difference was observed in *MCDA* mRNA levels between $Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice or between $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice.

MTP and *ApoB100* are key genes for VLDL secretion. Both *MTP* and *ApoB100* mRNA levels were significantly lower in $Lepr^{db/db}Smp30^{Y/-}$ mice than in $Lepr^{db/+}Smp30^{Y/+}$ and $Lepr^{db/+}Smp30^{Y/-}$ mice ($P<0.001$). Compared with $Lepr^{db/db}Smp30^{Y/+}$ mice, *MTP* mRNA expression levels in $Lepr^{db/db}Smp30^{Y/-}$ mice were decreased, although the change did not reach statistical significance. There was no significant difference in *ApoB100* mRNA levels between $Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice.

Interestingly, hepatic *LDLR* mRNA levels were significantly lower in $Lepr^{db/db}Smp30^{Y/-}$ mice than in $Lepr^{db/+}Smp30^{Y/+}$ ($P<0.05$) and $Lepr^{db/db}Smp30^{Y/+}$ ($P<0.05$) mice, but not $Lepr^{db/+}Smp30^{Y/-}$ mice. Hepatic VLDLR mRNA levels were significantly higher in $Lepr^{db/db}Smp30^{Y/-}$ mice than in $Lepr^{db/+}Smp30^{Y/+}$ ($P<0.001$) and $Lepr^{db/+}Smp30^{Y/-}$ mice ($P<0.001$), and were significantly lower than in $Lepr^{db/db}Smp30^{Y/+}$ mice ($P<0.05$). *CD36* mRNA levels were

A

	$Lepr^{db/+}Smp30^{Y/+}$	$Lepr^{db/+}Smp30^{Y/-}$	$Lepr^{db/db}Smp30^{Y/+}$	$Lepr^{db/db}Smp30^{Y/-}$
NASH activity score				
steatosis	0	0	2.8±0.2 [§,¶]	2.6±0.2 [§,¶]
inflammation	0	0	0.4±0.2	1.8±0.4 [†,‡,**]
ballooning	0	0	0	0
Total	0	0	3.2±0.4 [§,¶]	4.4±0.2 [§,¶,**]

B

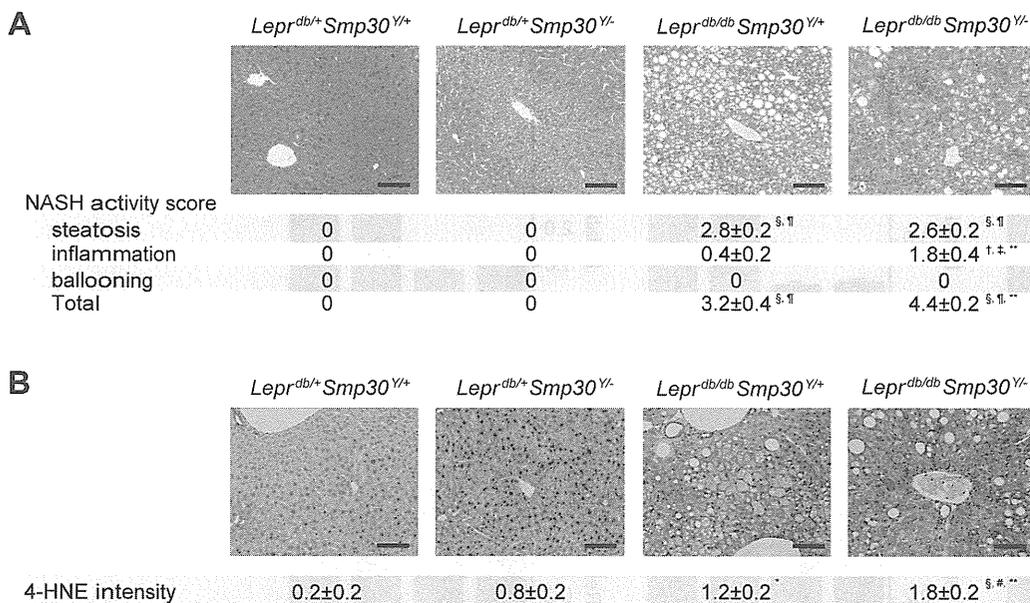|	$Lepr^{db/+}Smp30^{Y/+}$	$Lepr^{db/+}Smp30^{Y/-}$	$Lepr^{db/db}Smp30^{Y/+}$	$Lepr^{db/db}Smp30^{Y/-}$
4-HNE intensity	0.2±0.2	0.8±0.2	1.2±0.2	1.8±0.2 [§,#,**]

Figure 5. Increased steatosis, inflammation and oxidative stress in liver sections from $Lepr^{db/db}Smp30^{Y/-}$ mice. Representative images of (A) hematoxylin/eosin staining and (B) 4-HNE immunostaining in liver sections from $Lepr^{db/+}Smp30^{Y/+}$, $Lepr^{db/+}Smp30^{Y/-}$, $Lepr^{db/db}Smp30^{Y/+}$ and $Lepr^{db/db}Smp30^{Y/-}$ mice. Scale bar is 100 μm. $^*P<0.05$, $^†P<0.01$ and $^§P<0.001$ versus $Lepr^{db/+}Smp30^{Y/+}$, $^#P<0.05$, $^‡P<0.01$ and $^¶P<0.001$ versus $Lepr^{db/+}Smp30^{Y/-}$, $^{**}P<0.05$ versus $Lepr^{db/db}Smp30^{Y/+}$. Values are given as mean ± SEM of five animals.
doi:10.1371/journal.pone.0065698.g005

significantly higher in $Lepr^{db/db}Smp30^{Y/-}$ mice than in $Lepr^{db/+}Smp30^{Y/+}$ (P<0.001), $Lepr^{db/+}Smp30^{Y/-}$ (P<0.001) or $Lepr^{db/db}Smp30^{Y/+}$ (P<0.05) mice.

$Lepr^{db/db}Smp30^{Y/-}$ mice show increased endoplasmic reticulum (ER) stress

The high plasma sdLDL-cho, high liver TBARS content and low mRNA expression levels of fatty acid β oxidation and lipid secretion genes in $Lepr^{db/db}Smp30^{Y/-}$ mice prompted us to analyze hepatic levels of ER stress markers. No significant differences in spliced *XBP1* mRNA expression were observed among the four experimental mice groups (Fig. 7A). Phosphorylated eIF2α (phos-eIF2α) levels in $Lepr^{db/db}Smp30^{Y/-}$ mice were significantly (two-fold) higher (P<0.01) compared with $Lepr^{db/+}Smp30^{Y/+}$ mice (Fig. 7B). Also, there was a non-significant increase in phos-eIF2α levels in $Lepr^{db/db}Smp30^{Y/-}$ mice compared with $Lepr^{db/+}Smp30^{Y/-}$ or $Lepr^{db/db}Smp30^{Y/+}$ mice (Fig. 7B). On the other hand, CHOP levels were significantly higher in $Lepr^{db/db}Smp30^{Y/-}$ than in $Lepr^{db/+}Smp30^{Y/+}$ (P<0.01), $Lepr^{db/+}Smp30^{Y/-}$ (P<0.01) or $Lepr^{db/db}Smp30^{Y/+}$ (P<0.05) mice.

Discussion

The two-hit theory proposed by Day and James [22], in which the initial trigger is the hepatic accumulation of excessive fat, followed by the second hit of oxidative stress, is widely advocated as a pathogenic mechanism for NASH. Therefore, it is of great interest to study in greater detail the role of SMP30 in relation to the pathogenic mechanism for NAFLD/NASH in SMP30-KO mice on a $Lepr^{db/db}$ background. Interestingly, we observed first that SMP30 levels in $Lepr^{db/db}Smp30^{Y/+}$ mice were significantly lower than in $Lepr^{db/+}Smp30^{Y/+}$ mice, which suggests that this decrease is related to the development of obesity and obesity-related disorders in $Lepr^{db/db}Smp30^{Y/+}$ mice. The mechanism(s) of SMP30 reduction in $Lepr^{db/db}Smp30^{Y/+}$ mouse livers is unknown. Liver SMP30 protein levels were reported to decrease following

liver injury in animals treated with carbon tetrachloride [23], lipopolysaccharide (LPS) [24] or D-galactosamine/LPS [25]. Furthermore, we recently reported that 17β-estradiol attenuates saturated fatty acid diet-induced apoptotic liver injury in ovariectomized mice by up-regulating hepatic SMP30 [14]. Thus, the lipid deposition accompanied by increased oxidative and ER stress in $Lepr^{db/db}Smp30^{Y/-}$ mice might result from decreased liver SMP30 levels and in turn exacerbate liver damage via decreased Ca^{2+}-pumping activity and anti-oxidative effects of SMP30. Further study will be required to reveal the mechanisms of SMP30 suppression in obesity and obesity-related diseases.

A noteworthy finding of this study is that plasma levels of LDL-cho, in particular the smaller sized particles of Fr.no. 12 and Fr.no. 13 that correspond to small dense LDL-cho (sdLDL-cho) and the LDL-cho/HDL-cho ratio in $Lepr^{db/db}Smp30^{Y/-}$ mice, were significantly higher than those in $Lepr^{db/db}Smp30^{Y/+}$ mice, although plasma levels of T-cho in $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice were similar. A significantly increased LDL-cho/HDL-cho ratio was also found in $Lepr^{db/+}Smp30^{Y/-}$ mice compared with $Lepr^{db/+}Smp30^{Y/+}$ mice. These results indicate that the SMP30 deficiency contributes to increases in plasma sdLDL-cho and decreases in HDL-cho regardless of leptin receptor mutation followed by hyperphagia and obesity. In a human study an association between fatty liver and increased sdLDL-cho was reported [26–28]. Furthermore, we recently reported that in patients with histologically diagnosed NAFLD/NASH, serum sdLDL-cho levels in patients with NAS ≥5 were significantly higher than those in patients with NAS≤2, and sdLDL-cho was significantly and inversely correlated with hepatic SMP30 levels [29]. However, we do not presently have an explanation for the observed increase in sdLDL-cho in $Lepr^{db/db}Smp30^{Y/-}$ mice. In humans, TG-rich VLDL (large VLDL1) can be a precursor of sdLDL-cho, i.e., large VLDL1 particles are converted to sdLDL particles by cholesteryl ester transport protein (CETP) and hepatic lipase (HL), the levels of which are commonly increased in type 2 diabetes [30]. Unlike humans, however, mice do not express
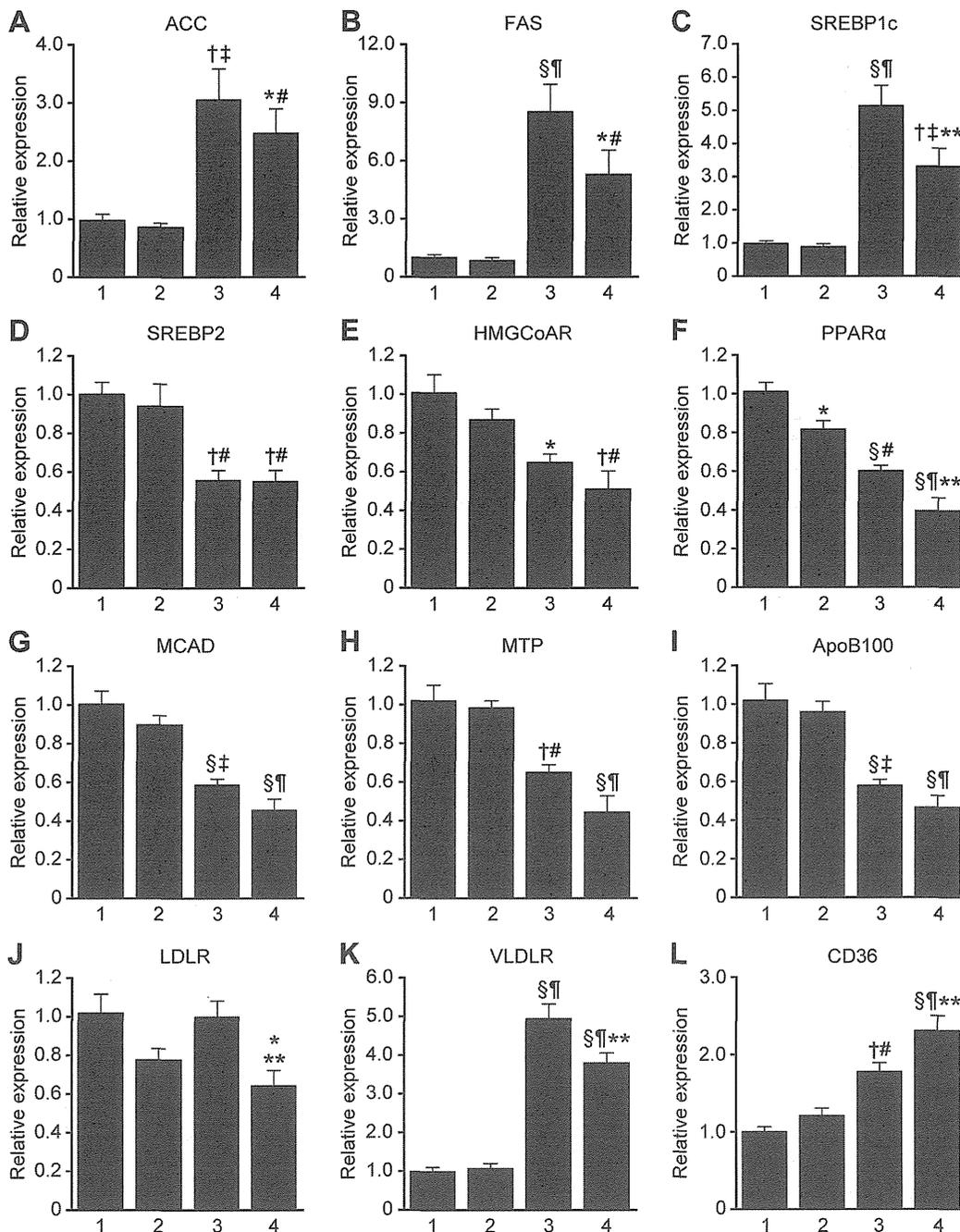
Figure 6. Altered hepatic expression of lipid/lipoprotein metabolism-related genes in $Lepr^{db/db}Smp30^{Y/-}$ mice. Gene expression levels of (A) ACC, (B) FAS, (C) SREBP1c, (D) SREBP2, (E) HMGCoAR, (F) PPARα, (G) MCAD, (H) MTP, (I) ApoB100, (J) LDLR (K) VLDLR, and (L) CD36 in the livers. Lane 1: $Lepr^{db/+}Smp30^{Y/+}$, lane 2: $Lepr^{db/+}Smp30^{Y/-}$, lane 3: $Lepr^{db/db}Smp30^{Y/+}$ and lane 4: $Lepr^{db/db}Smp30^{Y/-}$ mice. mRNA for each gene was measured using real time RT-PCR and normalized to β-actin. The values from $Lepr^{db/+}Smp30^{Y/+}$ mice were assigned a relative value of 1.0. Values are given as means ± SEM of five animals. $^*P<0.05$, $^\dagger P<0.01$ and $^\S P<0.001$ versus $Lepr^{db/+}Smp30^{Y/+}$, $^\#P<0.05$, $^\ddagger P<0.01$ and $^\P P<0.001$ versus $Lepr^{db/+}Smp30^{Y/-}$, $^{**}P<0.05$ versus $Lepr^{db/db}Smp30^{Y/+}$.
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CETP, and as such, cholesterol is mainly present in HDL. Qiu et al. reported that HL-deficient mice have sdLDLs, but TG enrichment was not observed in these mice [31]. In this study, no difference was observed in the mRNA expression of HL between $Lepr^{db/db}Smp30^{Y/-}$ and $Lepr^{db/db}Smp30^{Y/+}$ mice or between $Lepr^{db/+}Smp30^{Y/-}$ and $Lepr^{db/+}Smp30^{Y/+}$ mice (data not shown). Recently, we demonstrated that testosterone-deficient mice fed a high-fat diet showed markedly decreased serum TG and TG-

VLDL levels and markedly increased serum sdLDL-cho levels, likely due to altered expression of genes involved in hepatic assembly and lipid secretion [32]. Further work will be required to elucidate the molecular mechanism for this increase in sdLDL-cho in SMP30-KO mice.

An additional notable finding of this study was that although $Lepr^{db/db}Smp30^{Y/-}$ mice showed no advanced stage NASH including fibrosis, compared with $Lepr^{db/db}Smp30^{Y/+}$ mice $Lepr^{db/db}$

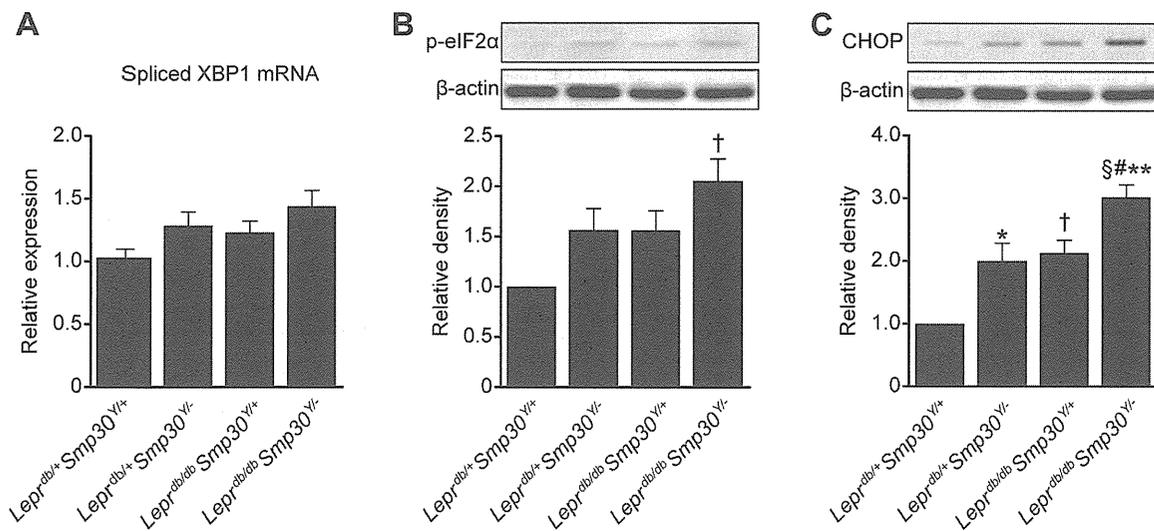– 226 –

Figure 7. Increase in endoplasmic reticulum (ER) stress in livers from *Lepr$^{db/db}$Smp30$^{Y/-}$* mice. (A) Spliced XBP1 mRNA expression, (B) Phosphorylated eIF2α protein and (C) CHOP protein levels in livers from the four experimental groups. Values are given as means ± SEM of five animals. *$P<0.05$, †$P<0.01$ and §$P<0.001$ *versus Lepr$^{db/+}$Smp30$^{Y/+}$*, #$P<0.05$ *versus Lepr$^{db/+}$Smp30$^{Y/-}$*, **$P<0.05$ *versus Lepr$^{db/db}$Smp30$^{Y/+}$*.
doi:10.1371/journal.pone.0065698.g007

Smp30$^{Y/-}$ mice had increased NAS activity and inflammation scores as well as enhanced oxidative stress. Hepatic steatosis results from an imbalance in lipid homeostasis in the liver, where fat uptake, *de novo* lipogenesis, fatty acid oxidation and fat export occur. Compared with *Lepr$^{db/db}$Smp30$^{Y/+}$* mice, RT-PCR of lipid homeostasis-related genes in the liver revealed that the expression levels of these genes, in particular *PPARα* and *SREBP-1c* (but not *CD36*), are reduced in *Lepr$^{db/db}$Smp30$^{Y/-}$* mice. CD36, a member of the class B scavenger receptor family of cell surface proteins, is abundantly expressed in monocytes/macrophages. Therefore, compared with *Lepr$^{db/db}$Smp30$^{Y/+}$* mice, the increase in *CD36* mRNA seen in *Lepr$^{db/db}$Smp30$^{Y/-}$* mice is likely a partial reflection of the increase in the number of inflammatory cells. *PPARα* mRNA levels were significantly lower not only in *Lepr$^{db/+}$Smp30$^{Y/-}$* mice compared to *Lepr$^{db/+}$Smp30$^{Y/+}$* mice, but also in *Lepr$^{db/db}$Smp30$^{Y/-}$* mice compared to *Lepr$^{db/db}$Smp30$^{Y/+}$* mice, suggesting that decreased hepatic *SMP30* mRNA expression is associated with mitochondrial and peroxisomal fatty acid β-oxidation.

Mitochondrial dysfunction is known to cause increases in oxidative stress, and indeed we showed that the levels of TBARS and 4-HNE rose in the livers of *Lepr$^{db/db}$Smp30$^{Y/-}$* mice. When SMP30-KO mice are fed a vitamin C-deficient diet they do not thrive and display symptoms of scurvy such as bone fractures and rachitic rosary before dying around three months after beginning the deficient diet. As such, *Lepr$^{db/+}$Smp30$^{Y/-}$* and *Lepr$^{db/db}$Smp30$^{Y/-}$* mice were given vitamin C-supplemented water in this study. Vitamin C is known to play an important role in the structure and function of mitochondria and endoplasmic reticulum [33]. Harrison *et al.* reported that vitamin E and vitamin C

treatment improves fibrosis but not necroinflammation in NASH patients [34]. Therefore, vitamin C supplementation would be one reason that fibrosis was not observed in *Lepr$^{db/db}$Smp30$^{Y/-}$* mice in this study.

In this study, *Lepr$^{db/+}$Smp30$^{Y/-}$* mice exhibited no phenotypes for lipid accumulation and mitochondrial damage in the liver as was previously reported [10]. However, in the previous study, mice were fed an autoclaved CRF-1 diet containing <55 mg of vitamin C per kg and tap water, and thus the liver vitamin C level in *Smp30$^{Y/-}$* mice was about 6% that of *Smp30$^{Y/+}$* mice [8]. Although lipid accumulation and mitochondrial damage were observed in livers of *Smp30$^{Y/-}$* mice at 12 months [10], it is unclear whether the phenotypes were caused by SMP30 deficiency (loss of unknown function except for vitamin C biosynthesis) and/or by vitamin C deficiency. Thus, the lack of steatosis in *Lepr$^{db/+}$Smp30$^{Y/-}$* mice might result from their younger age (24 weeks) or the vitamin C supplementation in the current study.

Although further studies will be required to define the exact molecular mechanism of the altered lipid homeostasis and liver damage caused by decreases in SMP30 levels, our data strongly suggest that SMP30 is closely associated with NAFLD pathogenesis and might be a possible therapeutic target for NAFLD.

Author Contributions

Conceived and designed the experiments: Y. Kondo GH NM H. Obayashi AI. Performed the experiments: Y. Kondo GH H. Okada TS MF NN MS JK TO Y. Kishimoto AA NM H. Obayashi AI. Analyzed the data: Y. Kondo GH H. Okada TS MF NN MS JK TO Y. Kishimoto AA NM H. Obayashi AI. Wrote the paper: Y. Kondo H. Obayashi AI.

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Clinicopathological features of liver injury in patients with type 2 diabetes mellitus and comparative study of histologically proven nonalcoholic fatty liver diseases with or without type 2 diabetes mellitus

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Abstract

Background The Japan Society of Diabetes Mellitus reported that the leading cause of death in patients with diabetes mellitus (DM) was chronic liver disease; however, there are limited studies investigating the cause of liver injury in these patients. Our study aimed to clarify the clinicopathological features of liver injury and the characteristics of nonalcoholic fatty liver disease (NAFLD) in DM patients.

Methods In total, 5,642 DM patients and 365 histologically proven NAFLD patients were enrolled. Clinical and laboratory parameters and liver biopsy results were, respectively, recorded and analyzed for the two sets of patients.

Results Positivity rates for Hepatitis B surface antigens (HBsAg) and anti-hepatitis C virus antibodies (anti-HCV Ab) were 1.7 and 5.1 %, respectively. The proportion of drinkers consuming 20–59 g and ≥60 g alcohol daily was 14.9 and 4.3 %, respectively. The percentage of DM patients with elevated serum alanine aminotransferase (ALT) levels (≥31 IU/L) was 28.6 %. Alcohol consumption had no significant effect on serum ALT levels. Seventy-two percent of HBsAg-positive patients were serum hepatitis B virus (HBV)-DNA negative, whereas 10 % exhibited high levels of the same (>4.0 log copies/ml).

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Thirty-eight percent of anti-HCV Ab-positive patients were serum HCV-RNA negative. Among the NAFLD patients, the frequencies of NASH and advanced stage NASH were significantly higher in male DM patients than in male patients without DM.

Conclusions Although HBsAg- and anti-HCV Ab-positivity rates were high in our Japanese DM patients, a majority of liver injuries could be associated with NAFLD/nonalcoholic steatohepatitis.

Keywords Nonalcoholic fatty liver disease ·
Nonalcoholic steatohepatitis · Diabetes mellitus · Hepatitis virus carrier · Alcoholic liver disease · Nationwide study

Abbreviations

HCC	Hepatocellular carcinoma
NAFLD	Nonalcoholic fatty liver disease
DM	Diabetes mellitus
NASH	Nonalcoholic steatohepatitis
HBV	Hepatitis B virus
HCV	Hepatitis C virus
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
FPG	Fasting plasma glucose
HOMA-IR	The homeostasis model assessment of insulin resistance index
HBsAg	Hepatitis B surface antigen
anti-HBc Ab	Anti-hepatitis B core antibody
anti-HCV Ab	Anti-hepatitis C virus antibody
HBV-DNA	Hepatitis B virus-deoxyribonucleic acid
HCV-RNA	Hepatitis C virus-ribonucleic acid
OR	Odds ratio
CI	Confidence interval

Introduction

As per the International Diabetes Federation, the number of diabetes mellitus (DM) sufferers rose to 366 million in 2011, representing 8.3 % of the global adult population, which is increasing in every country [1]. Worldwide, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer mortality [2]. HCC largely occurs in patients with chronic liver disease. Persistent hepatitis C virus (HCV) or hepatitis B virus (HBV) infections are the main causes of HCC; however, non-HCV- and non-HBV-associated HCC cases are increasing in Japan [3].

In 2007, the Japan Society of DM reported that the most frequent cause of death among 18,385 DM patients who died in hospitals during 1991–2000 was malignancy (34.1 %), followed by ischemic heart disease (10.2 %) and

cerebrovascular disease (9.8 %) [4]. Among the malignancies, HCC showed the highest frequency (8.6 %), followed by lung (5.3 %), pancreatic (4.8 %), and gastric cancer (3.5 %). Furthermore, the frequency of deaths caused by liver cirrhosis was 4.7 %, and in total, 13.3 % DM patients died of liver diseases. The cancer death rate in that study was quite different from that reported in the general Japanese population, in which lung (5.7 %), gastric (4.7 %), and colon (2.5 %) cancer occur with high frequencies [5]. Moreover, the death rate from liver diseases (13.3 %) was three times higher than that in the general Japanese population (HCC 3.2 %, liver cirrhosis 1.5 %, total 4.7 %) [6]. However, the incidences of HBV and HCV infection and the details of alcohol intake were not analyzed in that report.

The Japan Nonalcoholic Steatohepatitis (NASH) Study Group was founded in 2007 to investigate the cause of death in DM patients, the genetic factors in nonalcoholic fatty liver disease (NAFLD) patients, and the background of NASH-HCC patients [7]. This study focused on clarifying the cause of liver injury in Japanese DM patients and investigating the histological distribution of NAFLD in patients with and without DM.

Patients and methods

Patients

In total, 5,642 DM patients (3,238 males, 2,404 females) who visited nine DM clinics belonging to the Japan NASH Study Group (Saiseikai Suita Hospital; Kagoshima University Graduate School of Medical and Dental Sciences; Graduate School of Medicine, The University of Tokyo; Kanazawa University Graduate School of Medical Science; Department of Medicine, Asahikawa Medical College; Yamagata University Faculty of Medicine; Kyoto Prefectural University of Medicine; Okayama Saiseikai General Hospital; Fukui-ken Saiseikai Hospital) between January 2008 and December 2009 were enrolled in this observational study.

Three hundred and sixty-five NAFLD patients (182 males, 183 females) who visited Saiseikai Suita Hospital were enrolled in the histopathological study.

The study protocol was approved by the Human Ethics Committee of each participating hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Clinical and laboratory assessment

Demographic parameters, including age, sex, height, weight, and body mass index (BMI), and comorbidities, including alcohol consumption, hypertension, and dyslipidemia, were

recorded for all subjects in addition to the treatment administered for DM and the frequency of HCC occurrence. Clinical laboratory tests were conducted to measure aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), albumin, total cholesterol, triglyceride (TG), ferritin, uric acid, hemoglobin A1c, fasting plasma glucose (FPG), and insulin levels. The homeostasis model assessment of insulin resistance (HOMA-IR) index; platelet (PLT) count; and hyaluronic acid, type IV collagen 7S, hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibody (anti-HBc Ab), anti-HCV antibody (anti-HCV Ab), HBV-DNA, and HCV-RNA levels were also measured.

Blood samples were procured in the morning after overnight fasting. HOMA-IR was only calculated for subjects with FPG <140 mg/dL. HBV-DNA levels were measured by PCR (Amplicor HBV-DNA kit, Roche Diagnostics) or real-time PCR (TaqMan HBV-DNA kit, Roche Diagnostics) for HBsAg-positive, whereas HCV-RNA levels were measured by PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) or real-time PCR (TaqMan HCV-RNA kit, Roche Diagnostics) for anti-HCV Ab-positive patients.

Histopathological examination

In total, 365 patients (177 non-DM and 188 DM) at Saiseikai Suita Hospital fulfilled the criteria for NAFLD, namely serum HBsAg and anti-HCV Ab negativity, no alcohol consumption, and the absence of autoimmune liver diseases or hereditary liver injury. These patients underwent an ultrasound-guided liver biopsy using a 16G needle.

Specimens were fixed in formalin, embedded in paraffin, and subjected to hematoxylin–eosin, Masson trichrome, and Perl's iron staining. Histological features of samples were interpreted according to a method described by Matteoni et al [8]. NASH stage was classified according to Brunt's classification [9].

Statistical analysis

All statistical analyses were performed using SPSS for Windows (SPSS Japan Inc.). Data were summarized by frequency for categorical variables and mean ± standard deviation (SD) for continuous variables. The Chi-square test was used to determine the differences between categorical variables. Student's t test was used to compare means of continuous valuables with equal variance, and the Mann–Whitney U test was used for non-normally distributed variables. The Cochran–Armitage test was used to study the trend of continuous variables. Forward stepwise logistic regression analysis was used to identify independent variables related to elevated serum ALT ($\geq$31 IU/L)

levels. A p value of <0.05, obtained by a two-tailed test, was considered statistically significant.

Since there is no official report on the HBV and HCV carrier rate in the general Japanese population, we utilized blood donor data for comparison with our patients [10].

Results

Baseline characteristics

The mean age and BMI of male and female DM patients was 62.2 and 64.8 years and 24.5 and 24.7 kg/m^2, respectively (Table 1). Hypertension and dyslipidemia occurred in 51.0 and 63.3 % of DM patients, respectively. Respective DM treatment types in DM patients were as follows: no medication, 20.5 %; oral drugs, 47.7 %; insulin, 28.9 %; and oral drugs and insulin, 2.8 % (Table 2).

Mean ALT level was significantly higher in males (30.6 IU/L) than in females (Table 1). Abnormal serum ALT levels ($\geq$31 IU/L) were found in 28.6 % of DM patients (males 32.8 %, females 23.0 %). When the healthy upper limit of abnormal serum ALT level in females was defined as 20 IU/L according to Prati et al.'s [11] criteria, the frequency of abnormal ALT ($\geq$21 IU/L) levels in females was 43 %. The mean PLT count was 20.8 × 10^4/μL in males and 21.9 × 10^4/μL in females. Mean values of other clinical laboratory tests are shown in Table 1.

Prevalence of HBV and HCV infection and drinking and their effects on liver function tests

HBsAg positivity was detected in 1.7 % of DM patients (M 1.8 %, F 1.6 %) (Table 2); this was significantly higher than that (total 0.9 %, M 1.0 %, F 0.7 %) detected in 1.7 million blood donors aged >40 years (p < 0.001). For both sexes, the HBsAg detection rate was significantly higher in DM patients than in blood donors in the 50- to 59- and 60- to 69-year age groups (p < 0.05) (Fig. 1). There were no significant differences in serum AST, ALT, and GGT levels between HBsAg-positive and HBsAg-negative DM patients of both sexes.

Seventy-two percent of HBsAg-positive patients (M 69 %, F 79 %) demonstrated HBV-DNA negativity (<2.6 log copies/ml) (Table 3). Of the HBsAg-positive patients, only 10 % showed high serum HBV-DNA levels ($\geq$4.0 log copies/ml); these could be HBV infection-induced liver injury cases. Mean values of age, serum ALT level, and PLT counts in HBV-DNA-negative HBV carriers were 63.6 years, 25.3 IU/L, and 20.5 × 10^4/μL, respectively. HBV-DNA-negative HBV carriers were older and exhibited lower ALT levels and higher PLT counts; however, the differences were not significant.

Table 1 Backgrounds of diabetes mellitus patients (1)

Characteristic	Total subjects		Males		Females		
	n	M ± SD	n	M ± SD	n	M ± SD	p
Age (years)	5,510	63.3 ± 12.7	3,164	62.2 ± 12.5	2,346	64.8 ± 12.9	<0.001
BMI (kg/m²)	5,173	24.6 ± 4.7	2,987	24.5 ± 4.2	2,186	24.7 ± 5.2	0.629
Aspartate aminotransferase (IU/L)	5,568	26.4 ± 17.2	3,188	27.1 ± 18.0	2,380	25.5 ± 15.9	<0.001
Alanine aminotransferase (IU/L)	5,569	28.2 ± 24.5	3,190	30.6 ± 26.9	2,379	24.9 ± 20.5	<0.001
GGT (IU/L)	5,476	48.3 ± 72.5	3,131	59.6 ± 86.7	2,345	33.1 ± 42.9	<0.001
Albumin (g/dL)	5,031	4.2 ± 0.4	2,869	4.2 ± 0.5	2,162	4.1 ± 0.4	<0.001
Platelet (×10⁴/µL)	5,419	21.3 ± 6.1	3,112	20.8 ± 6.0	2,307	21.9 ± 6.1	<0.001
Fasting plasma glucose (FPG; mg/dL)	5,123	152.7 ± 61.7	2,945	156.0 ± 63.9	2,178	148.3 ± 58.2	<0.001
HbA1c (%)	5,479	7.2 ± 1.7	3,143	7.2 ± 1.7	2,336	7.2 ± 1.6	0.744
HOMA-IR (FPG <140)	1,005	2.55 ± 2.60	570	2.51 ± 2.59	435	2.61 ± 2.60	0.209
Total cholesterol (mg/dL)	5,260	195.1 ± 39.5	3,016	191.6 ± 40.0	2,244	199.6 ± 38.5	<0.001
Triglycerides (mg/dL)	5,443	136.3 ± 102.7	3,119	145.1 ± 111.9	2,324	124.5 ± 87.5	<0.001
Hyaluronic acid (ng/mL)	559	74.5 ± 98.6	319	59.3 ± 73.0	240	94.6 ± 122.1	<0.001
Type 4 collagen 7S (ng/mL)	474	4.9 ± 2.0	269	4.8 ± 2.0	205	4.9 ± 1.9	0.544
Ferritin (ng/mL)	1,838	142.0 ± 157.0	1,084	171.9 ± 174.9	754	99.1 ± 114.1	<0.001
Uric acid (mg/dL)	3,645	5.4 ± 1.5	2,043	5.7 ± 1.4	1,602	4.9 ± 1.4	<0.001

Results are shown as mean ± SD

GGT gamma glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment of insulin resistance

Table 2 Backgrounds of diabetes mellitus patients (2)

Characteristic	Total subjects		Males		Females		
	n	Positive (%)	n	Positive (%)	n	Positive (%)	p
Hepatitis B surface antigen	4,867	83 (1.7 %)	2,796	50 (1.8 %)	2,071	33 (1.6 %)	0.604
Anti-hepatitis B core antibody	3,211	958 (29.8 %)	1,824	572 (31.4 %)	1,387	386 (27.8 %)	0.030
Anti-hepatitis C virus antibody	4,877	247 (5.1 %)	2,812	144 (5.1 %)	2,065	103 (5.0 %)	0.834
Alcohol	4,443		2,554		1,889		<0.001
<20 g/day		3589 (80.8 %)		1769 (69.3 %)		1820 (96.3 %)	
20–59 g/day		661 (14.9 %)		609 (23.8 %)		52 (2.8 %)	
≥60 g/day		193 (4.3 %)		176 (6.9 %)		17 (0.9 %)	
Hypertension	4,936	2515 (51.0 %)	2,841	1419 (49.9 %)	2,095	1096 (52.3 %)	0.100
Dyslipidemia	5,423	3434 (63.3 %)	3,091	1882 (60.9 %)	2,332	1552 (66.6 %)	<0.001
Diabetes mellitus (intervention)	5,227		3,013		2,214		0.002
None		1072 (20.5 %)		629 (20.9 %)		443 (20.0 %)	
Oral drugs		2495 (47.7 %)		1489 (49.4 %)		1006 (45.4 %)	
Insulin		1513 (28.9 %)		810 (26.9 %)		703 (31.8 %)	
Oral drugs + insulin		147 (2.8 %)		85 (2.8 %)		62 (2.8 %)	
Hepatocellular carcinoma	4,700	67 (1.4 %)	2,696	48 (1.8 %)	2,004	19 (0.9 %)	0.017

Anti-HCV Ab positivity was detected in 5.1 % (M 5.1 %, F 5.0 %) of DM patients; this rate was significantly higher than that (total 1.0 %, M 1.1 %, F 1.0 %) in blood donors of every age group of both sexes ($p < 0.001$), except for females aged 50–59 years (Table 2; Fig. 2). ALT and GGT levels were significantly higher in male anti-HCV Ab-positive patients than in their negative counterparts ($p < 0.001, p < 0.05$) (Fig. 2). For both sexes,

Fig. 1 Prevalence of HBV infection and the effect of HBV infection on laboratory tests in DM patients. **a** Prevalence of HBV infection in blood donors and DM patients. **b** The effect of HBV infection on laboratory tests in DM patients. There were no significant differences in serum AST, ALT, and GGT levels between HBsAg-positive and HBsAg-negative DM patients of both sexes. *Error bars* SD
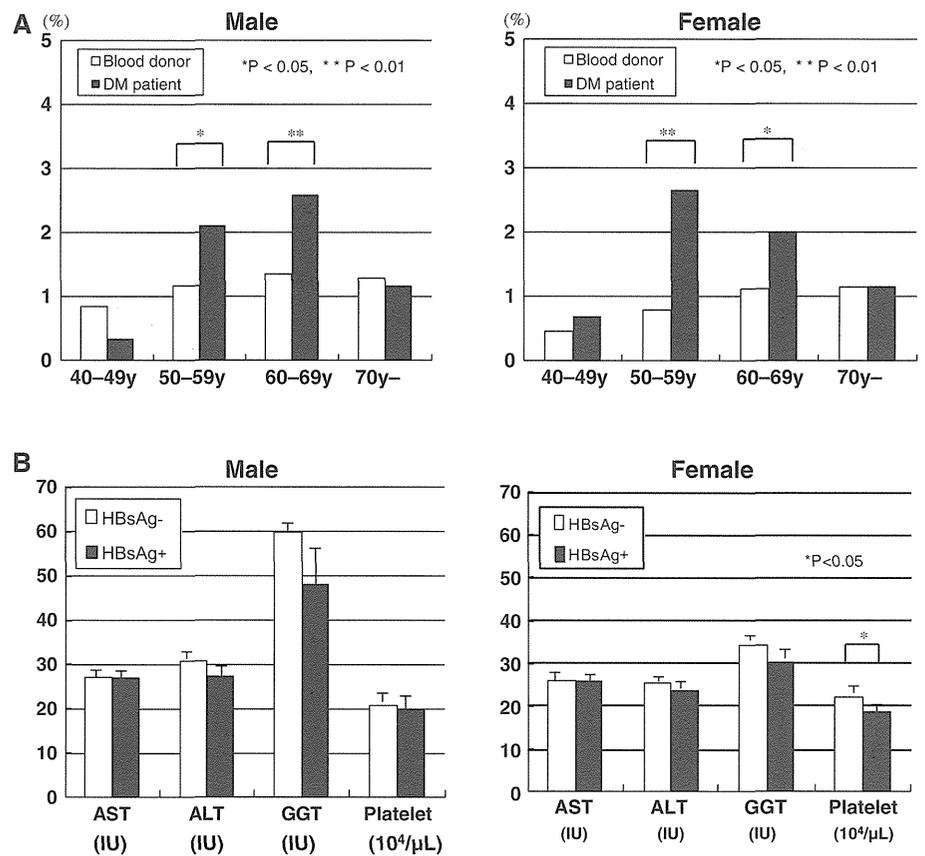

Table 3 Serum HBVDNA and HCVRNA levels, age, serum ALT level, and platelet (PLT) counts in HBsAg-positive patients and anti-HCV Ab-positive patients

	% (n)	Mean age (years)	Mean ALT levels (IU/L)	Mean PLT count($\times 10^4$/μL)
Serum HBV-DNA[a]				
Negative (<2.6 log copy/ml)	72 (29)	63.6	25.3	20.5
Positive (=2.6 log copy/ml)	28 (11)	55.6	28.0	18.5
=2.6<4.0	18 (7)	61.9	26.6	18.9
=4.0	10 (4)	50.0	30.5	17.8
Serum HCV-RNA[b]				
Negative (<2.7 log IU/ml)	38 (57)	67.2	28.2	17.7
Positive (=2.7 log IU/ml)	62 (91)	67.4	51.7	15.3
=2.7<5.0	3 (4)	65.1	28.0	16.4
=5.0	59 (87)	67.5	52.7	15.2

[a] Results are presented as either frequency or mean in 40 HBsAg-positive patients

[b] Results are presented as either frequency or mean in 148 anti-HCV Ab-positive patients

the PLT count was significantly lower in anti-HCV Ab-positive DM patients than in their negative counterparts ($p < 0.001$).

Thirty-eight percent of anti-HCV Ab-positive patients (M 36 %, F 42 %) demonstrated HCV-RNA negativity (Table 3), and 96 % of HCV-RNA-positive patients exhibited high serum HCV-RNA levels (≥5.0 log IU/ml). Serum ALT levels in anti-HCV Ab-positive patients with

HCV-RNA positivity and those with HCV-RNA negativity were 51.7 ± 39.7 and 28.2 ± 18.1 IU/L, respectively, whereas those in anti-HCV Ab-negative patients were 27.7 ± 22.8 IU/L. Serum ALT levels were significantly higher in HCV-RNA-positive patients than in HCV-RNA-negative patients ($p < 0.001$).

The proportion of DM patients consuming >60 g and 20–59 g alcohol daily was 4.3 % (M 6.9 %, F 0.9 %) and

Fig. 2 Prevalence of HCV infection and the effect of HCV infection on laboratory tests in DM patients. **a** Prevalence of HCV infection in blood donors and DM patients. **b** The effect of HCV infection on laboratory tests in DM patients. *GGT* gamma glutamyl transpeptidase. ALT and GGT levels were significantly higher in male anti-HCV Ab-positive patients than in their negative counterparts. *Error bars* SD
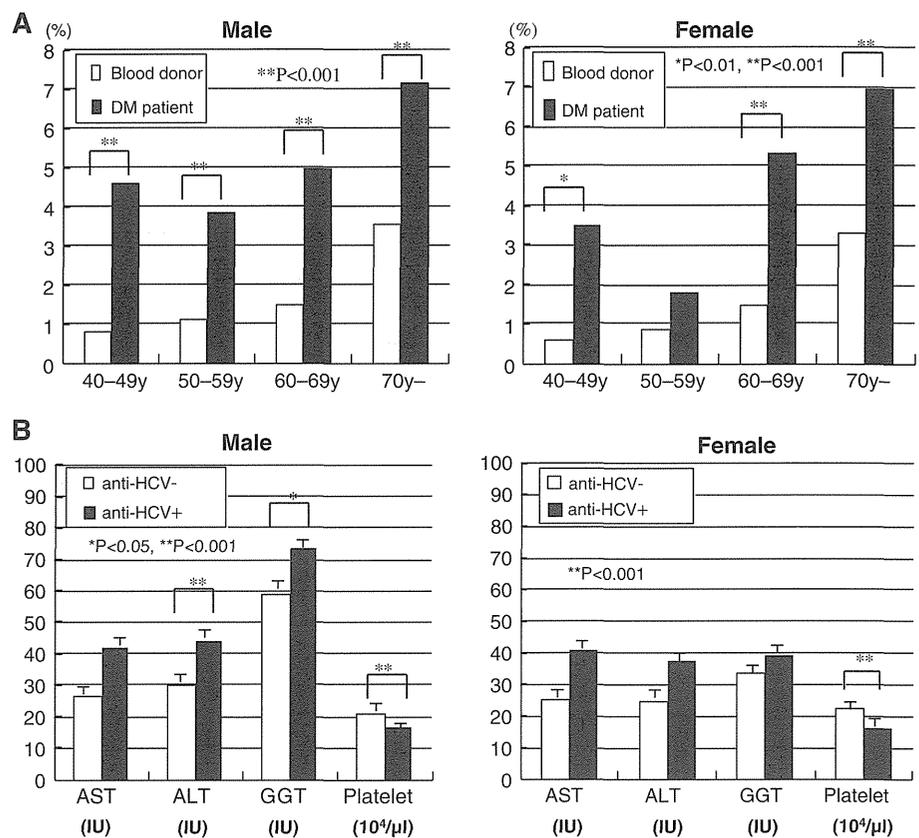
14.9 % (M 23.8 %, F 2.8 %), respectively (Table 2). The highest percentage of drinkers were males in the 60- to 69-year age group and females in the <40-year age group. Male drinkers consuming >60 g alcohol daily had significantly higher serum AST and GGT levels compared with nondrinkers (patients consuming <20 g of daily alcohol intake) ($p < 0.001$). Serum ALT levels in drinkers consuming >60 g alcohol daily were comparable with those in nondrinkers. Drinkers of both sexes consuming 20–59 g alcohol daily had significantly higher serum GGT levels ($p < 0.001$) (Fig. 3).

Factors related to serum ALT levels

With increasing age in both sexes, the number of DM patients with elevated serum ALT levels and high BMI decreased, whereas those with decreased PLT counts increased. The number of DM patients with elevated serum ALT levels increased with increasing BMI in both sexes (Fig. 4).

A forward stepwise logistic regression model yielding odds ratios (ORs) and 95 % confidence intervals (CIs) was used to analyze the factors related to elevated serum ALT levels. The model included BMI, age, drinking status, HBsAg status, anti-HCV Ab status, PLT count, hypertension status, and dyslipidemia status as independent

variables. The odds ratio shown indicates the change in odds for one SD increase in each variable.

Multivariate analysis showed that age (M: OR 0.674, CI 0.613–0.741; $p < 0.001$; F: OR 0.767, CI 0.683–0.861; $p < 0.001$), PLT count (M: OR 0.806, CI 0.732–0.886; $p < 0.001$, F: OR 0.714, CI 0.632–0.808, $p < 0.001$), anti-HCV Ab status (M: OR 1.321, CI 1.218–1.433; $p < 0.001$; F: OR 1.232, CI 1.117–1.359; $p < 0.001$), and BMI (M: OR 1.509, CI 1.374–1.657; $p < 0.001$; F: OR 1.487, CI 1.330–1.663; $p < 0.001$) were significantly associated with elevated serum ALT levels (Table 4).

For both sexes, AST and ALT levels were similar in drinkers consuming 20–59 g alcohol daily and those consuming <20 g alcohol daily (Fig. 3). After eliminating HBV-positive patients and/or HCV carriers and heavy drinkers consuming >60 g alcohol daily, the number of male, female, and total DM patients with elevated serum ALT levels were 33.4, 23.3, and 28.3 %, respectively. These values were comparable with those in all DM patients, including those with hepatitis and/or those consuming alcohol (M 32.8 %, F 23.0 %, total 28.6 %).

Liver histology in DM patients

The median age of histologically proven, DM- ($n = 87$) and non-DM-associated ($n = 95$) male NAFLD patients

Fig. 3 Drinking habits and the effect of alcohol consumption on laboratory tests in DM patients. a Drinking habits in individual age. b The effect of alcohol consumption on laboratory tests in DM patients. *GGT* gamma glutamyl transpeptidase. Serum ALT levels in drinkers consuming >60 g alcohol daily were comparable with those in nondrinkers. *Error bars* SD
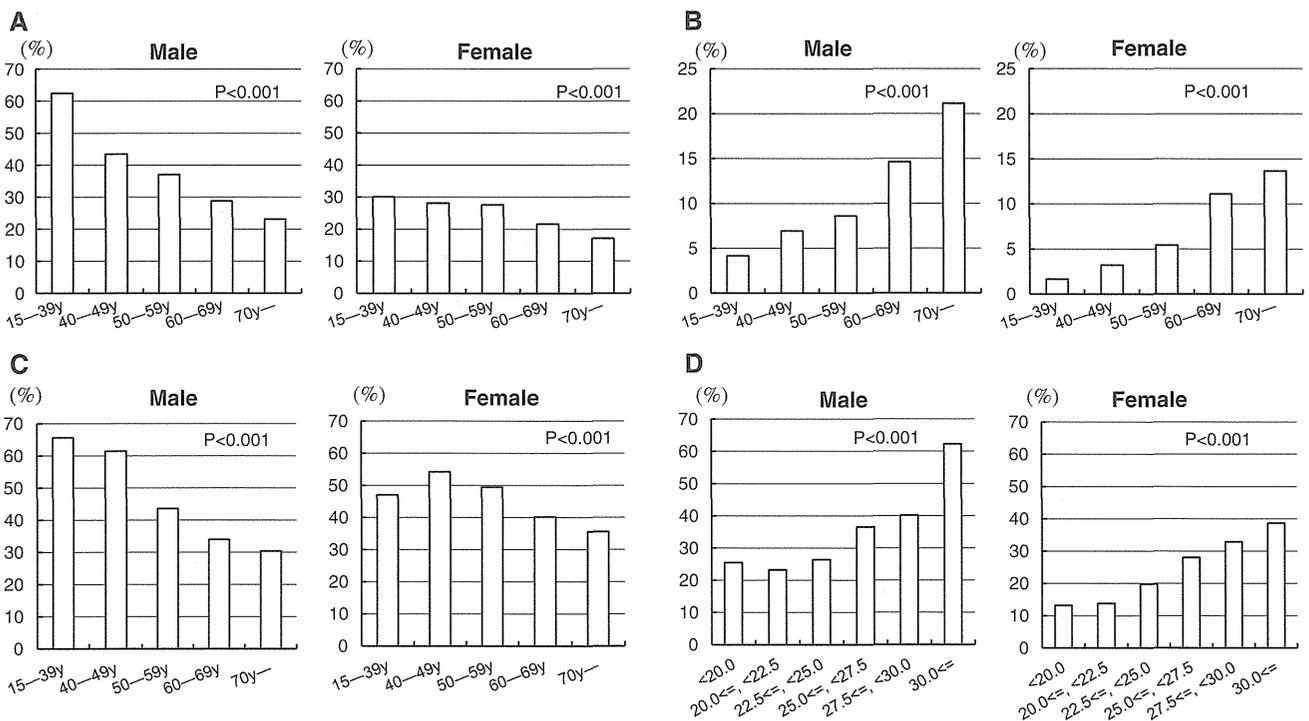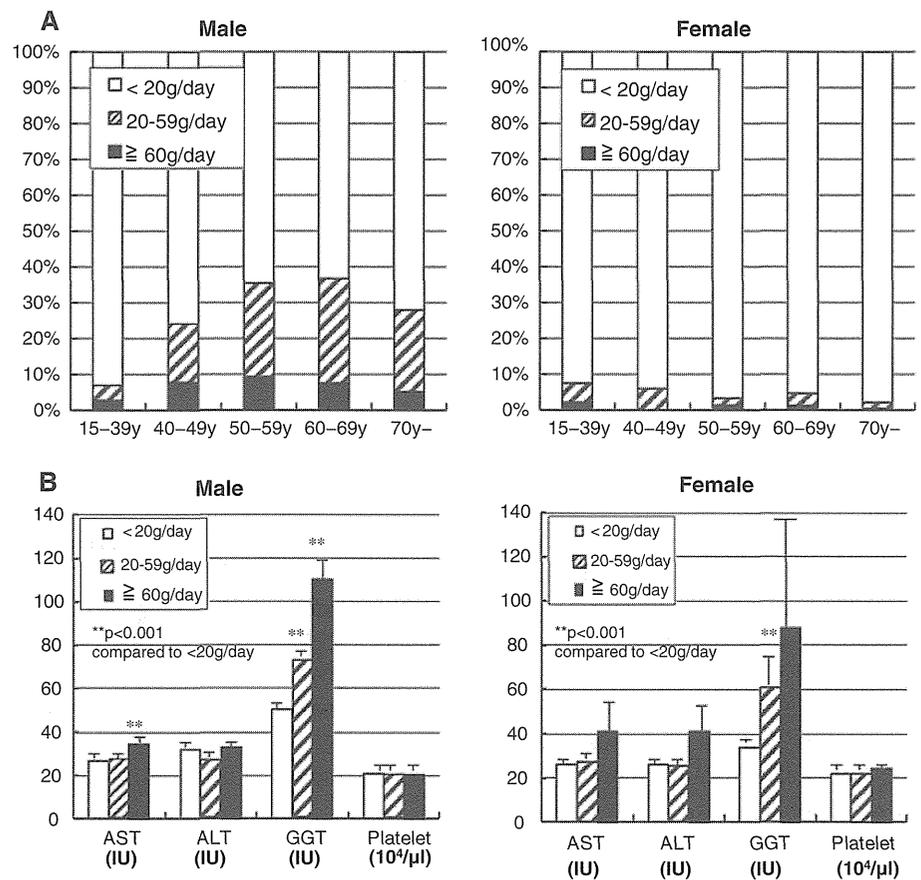

Fig. 4 Influence of age on the ratio of patients with elevated serum ALT level, decreased PLT count and abnormal BMI, and the relationship between BMI and the ratio of patients with elevated serum ALT level. a The ratio of patients with elevated serum ALT level ($\geq$31 IU/L). b The ratio of patients with decreased PLT count ($<15 \times 10^4/\mu$L). c The ratio of patients with abnormal BMI ($\geq$25). d The relationship between BMI and the ratio of patients with elevated serum ALT level ($\geq$31 IU/L)

Table 4 Multivariate analysis to identify independent variables related to elevated serum ALT level (≥31 IU/L)

	Regression coefficient	Standard error	Odds ratio	95 % confidence interval	p
Males					
Age	−0.394	0.048	0.674	0.613–0.741	<0.001
Platelet	−0.216	0.049	0.806	0.732–0.886	<0.001
Anti-hepatitis C virus	0.278	0.042	1.321	1.218–1.433	<0.001
Body mass index	0.411	0.048	1.509	1.374–1.657	<0.001
Females					
Age	−0.265	0.059	0.767	0.683–0.861	<0.001
Platelet	−0.336	0.063	0.714	0.632–0.808	<0.001
Anti-hepatitis C	0.208	0.050	1.232	1.117–1.359	<0.001
Body mass index	0.397	0.057	1.487	1.330–1.663	<0.001

was 60 and 45 years, respectively; in corresponding females, the median age was 66 ($n = 101$) and 61 years ($n = 82$), respectively. No significant difference was noted in BMI between DM and non-DM NAFLD patients (M 26.0 and 27.5 kg/m^2, respectively; F 26.0 and 27.0 kg/m^2, respectively). Male NAFLD patients without DM were significantly younger than those with DM ($p < 0.001$).

NAFLD patients were classified according to Matteoni's classification. Type 1, 2, 3, and 4 cases were 14 (16 %), 14 (16 %), 4 (5 %), and 55 (63 %), respectively, among male DM patients and 28 (29 %), 23 (24 %), 11 (12 %), and 33 (35 %), respectively, among male non-DM patients. Type 1, 2, 3, and 4 cases were 16 (16 %), 8 (8 %), 4 (4 %), and 73 (72 %), respectively, among female DM patients and 9 (11 %), 9 (11 %), 7 (9 %), and 57 (69 %), respectively, among female non-DM patients (Fig. 5). The frequency of Type 4 NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.001$). The rate of Type 4 NASH was high in both female DM and non-DM patients.

In total, 244 (M 103, F 141) NASH patients were classified according to Brunt's classification. The number of patients with stage 0 (Matteoni Type 3), 1, 2, 3, and 4 were 2 (3 %), 27 (46 %), 10 (17 %), 17 (29 %), and 3 (5 %), respectively, among male DM patients and 10 (23 %), 16 (36 %), 8 (18 %), 9 (21 %), and 1 (2 %), respectively, among male non-DM patients. Stage 0, 1, 2, 3, and 4 cases were 4 (5 %), 25 (33 %), 24 (31 %), 13 (17 %), and 11 (14 %), respectively, among female DM patients and 4 (6 %), 28 (44 %), 20 (31 %), 10 (16 %), and 2 (3 %), respectively, among female non-DM patients (Fig. 6). The frequency of advanced stage NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.05$). The rate of Stage 4 NASH was higher in female DM patients than in female non-DM patients; however, the difference was not significant ($p = 0.198$).
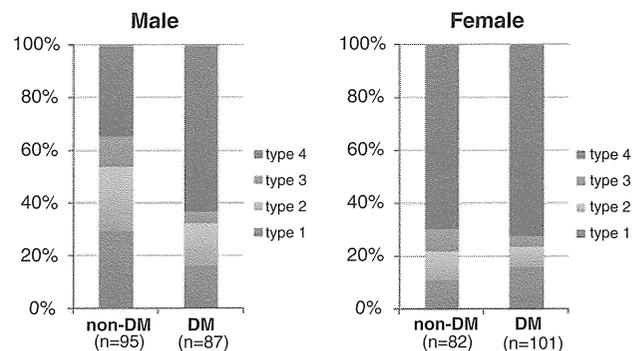

Fig. 5 Distribution of Matteoni's type classification in individual status of glucose metabolism among NAFLD patients. The frequency of type 4 NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.001$). The rate of type 4 NASH was high in both female DM and non-DM patients
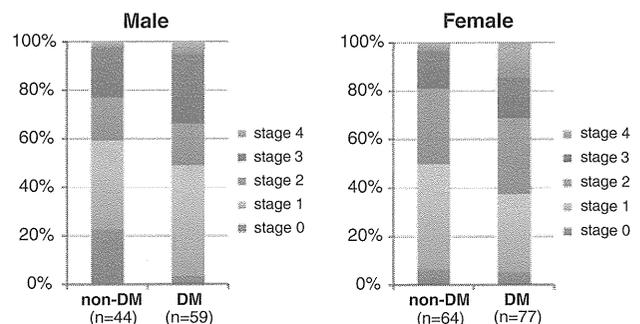

Fig. 6 Distribution of Brunt's stage in individual status of glucose metabolism among NASH patients. The frequency of advanced stage NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.05$). The rate of stage 4 NASH was higher in female DM patients than in female non-DM patients; however, the difference was not significant ($p = 0.198$)

HCC incidence in DM patients

In total, 67 (M 48, F 19) HCC cases (1.4 %) were reviewed (Table 2). HCC incidence was significantly higher in males