RESEARCH

Overexpression of the *Lias* gene attenuates hepatic steatosis in *Lepr^{db/db}* mice

Guangcui Xu¹, Tingting Yan¹, Qiang Peng¹, Haibin Li¹, Weidong Wu¹, Xianwen Yi² and Yingzheng Zhao¹¹School of Public Health, Xinxiang Medical University, Xinxiang, Henan Province, China²Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina, USACorrespondence should be addressed to X Yi or Y Zhao: xyi2000@med.unc.edu or yingzhengzhao@126.com

Abstract

Oxidative stress is proposed to be involved in nonalcoholic fatty liver disease (NAFLD). However, antioxidant therapy results in controversial outcomes. Therefore, we generated a new antioxidant/NAFLD mouse model, *Lias^{High/High}Lepr^{db/db}* mice, by crossbreeding *Lepr^{db/db}* mice, an obesity mouse model, with *Lias^{High/High}* mice, generated by overexpression of lipoic acid synthase gene (*Lias*) and having increased endogenous antioxidant capacity, to investigate whether the new model could block the development of NAFLD. We have systemically characterized the novel model based on the main features of human NAFLD, determined the impact of enhanced endogenous antioxidant capacity on the retardation of NAFLD and elucidated the underlying mechanisms using various biological and pathological methods. We found that *Lias^{High/High}Lepr^{db/db}* mice ameliorated many pathological changes of NAFLD compared with the control. In particular, *Lias^{High/High}Lepr^{db/db}* mice displayed the improved liver mitochondrial function, reflecting the decline of mitochondrial microvesicular steatosis, and reduced oxidative stress, which mainly contributes to the alleviation of pathologic alterations of the NAFLD progression. Our new model shows that mitochondrial dysfunction is a major pathogenesis for liver steatosis. Overexpression of *Lias* gene effectively reduces oxidative stress and protects mitochondria, and consequently attenuates NAFLD/NASH.

Key Words

- ▶ NAFLD
- ▶ mouse model
- ▶ lipoic acid
- ▶ antioxidant
- ▶ oxidative stress

Journal of Endocrinology
(2021) **248**, 119–131

Introduction

In association with the obesity pandemic, the prevalence of nonalcoholic fatty liver disease (NAFLD) and its progressive form, nonalcoholic steatohepatitis (Sanyal *et al.* 2010), is rising in the whole world and is estimated to reach 30% of all adults in developed countries (Pais *et al.* 2016, Wong & Wong 2016, Younossi *et al.* 2016). To date, the pathogenic mechanism of NAFLD/NASH has not been fully clarified. Therefore, none of drugs has been approved by FDA for NAFLD/NASH so far (Filozof *et al.* 2015).

According to the ‘two-hit theory’, oxidative stress is regarded as the ‘second hit’ that is required for the

development of NAFLD (Day & James 1998). Antioxidant therapy should represent a logical therapeutic strategy for the treatment of NAFLD. However, antioxidant therapies have produced controversial results in clinical trials (Singal *et al.* 2011). For example, vitamin E supplementation prevents fibrogenesis, reduces fatty liver and inflammation in NAFLD/NASH (Vajro *et al.* 2004, Caldwell & Lackner 2017). N-acetylcysteine (NAC) found improvement in liver enzymes in patients with NASH (Pamuk & Sonsuz 2003). On the other hand, other studies using vitamin E failed to show any beneficial effect on ALT, or liver fibrosis (Groenbaek *et al.* 2006, Sanyal *et al.* 2010).

The study of human NAFLD is hampered by the slow development of the disease. Thus, mouse models are indispensable tools to elucidate pathogenic mechanisms, not only to evaluate the pathogenesis and progression of NAFLD, and but test the therapeutic effects of various potential drugs. Until now, none of antioxidant/NAFLD model is available. To fill this gap, we have generated a unique antioxidant-NAFLD mouse model by mating *Lias*^{High/High} antioxidant mouse model with *Lepr*^{db/db} mice. *Lepr*^{db/db} mice have a defect in leptin receptor and generate many of the metabolic disturbances present in NAFLD patients (Solis-Munoz *et al.* 2011). The *db/db* animals are obese, present insulin resistance or diabetes, and develop hepatic steatosis. In addition, they may develop NASH after a second stimulus such as the intake of trans-fat or a methionine and choline-deficient (MCD) diet. When *db/db* mice are fed a MCD diet, they can develop significant liver fibrosis (Sahai *et al.* 2004, Wortham *et al.* 2008). It is possible that oxidative stress may serve as a second stimulus for the development of NAFLD into NASH. The phenotype of the *db/db* mice better simulates the condition of human metabolic syndrome in many circumstances. However, these mice are limited because they do not spontaneously develop liver fibrosis or NASH without a second stimulus (Takahashi 2012, Ka *et al.* 2017). It is possible that oxidative stress can work as the second stimulus to induce inflammation and/or liver fibrosis. It is well-known that mitochondria are the major cellular source of reactive oxygen species (Lindquist *et al.* 2018) and are the most immediate target of ROS. We postulated that excess ROS mainly produced from mitochondrial dysfunction due to a defective cellular antioxidant systems led to the 'second hit' for the development of NAFLD. We selected *Lias* mice for the creation of an antioxidant mouse model according to our hypothesis and several unique features of α -lipoic acid (LA) (Yi & Maeda 2005, Liu 2008, Tanaka *et al.* 2015, Namazi *et al.* 2018).

Lipoic acid (LA) is the product of lipoic acid synthetase (*Lias*) and a strong antioxidant. It is also a coenzyme of two enzymes (pyruvate dehydrogenase complex and α -ketoglutarate dehydrogenase complex) involved in energy metabolism in mitochondria (Dorsam & Fahrner 2016). LA plays an important role in the antioxidant defense system of the body. LA has the unique characteristics. For example, it can eliminate various free radicals in the body, enhance the antioxidant capacity through transcriptional regulation, possibly protect mitoquinone (MitoQ10) and recycle other antioxidants such as vitamin C and E, MitoQ10 and glutathione. The elimination of *Lias* gene will lead to the

embryo stop development, indicating that endogenous LA plays an irreplaceable role in antioxidant activity and energy regulation (Yi & Maeda 2005). Previous studies have repeatedly proved that both exogenous and endogenous lipoic acids significantly changed the level of antioxidation and the development of atherosclerosis (Yi & Maeda 2006, Yi *et al.* 2012).

Lias^{High/High} mice harbor overexpressed *Lias* gene and the genetic engineering strategy of *Lias*^{High/High} mice was based on genetic modification of the 3'-UTR of lipoic acid synthase (*Lias*) mRNA (Park *et al.* 2008, Xu *et al.* 2016). The Western blot result confirmed significantly increased *Lias* protein expression in *Lias*^{High/High} mice (~160% of *Lias* gene expression), accompanied by correspondingly increased antioxidant capacity and decreased oxidative stress compared to WT and consequently affects the development of these diseases (Xu *et al.* 2016). In this study, we characterized the antioxidant/NAFLD model, and showed that overexpression of *Lias* gene increased mitochondrial antioxidant capacity and improved NAFLD in *Lias*^{High/High}*Lepr*^{db/db} mice.

Materials and methods

Animals

Lepr^{db/+} heterozygous mice with the C57B6/J background were purchased from the Jackson Laboratory (Stock number: 000697, Bar Harbor). Animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Xinxiang Medical University (protocol number XYLL-2017086, approved 3 March 2017).

Lias^{High/High}*Lepr*^{db/db} mice were examined starting at 4 weeks of age. Age- and sex-matched *Lias*^{+/+}*Lepr*^{db/db} littermates served as controls. There were a total of 10 male and 7–9 female mice per group. All animals were fed a normal chow (produced according to NIH-07 standard and meet the hygienic standard of experimental animal compound feed (GB 14924.2)) with 4% fat and 20.5% protein content (Puluteng Inc. Shanghai, China). Water was provided *ad libitum*. The mice were housed in a 12 h light:12 h darkness cycle with ambient room temperatures of 20–25°C and relative humidity of 60–70%. At the 28th week, the mice were sacrificed after anesthetized with isoflurane. The blood was collected from the femoral artery and the livers were separated from the animal for weighing. A portion of the liver was fixed in 4% paraformaldehyde for histological evaluation.

The plasma and liver samples were stored at -80°C until they were examined.

Genotyping by TaqMan probe fluorescence quantitative PCR

The *Lepr^{db/db}* mouse has a G $\rightarrow$ T point mutation in the leptin receptor gene. Because homozygous mice are infertile, efficient maintenance of this strain requires an accurate genotyping strategy to detect heterozygotes. However, commonly used methods are time-consuming and unstable with the requirement of restriction enzyme digestion. Thus, we adapted a new method to efficiently identify the genotype. In brief, SNP database website (<http://www.ncbi.nlm.nih.gov/snp>), *Lepr^{db/+}* gene sequence (gene sequence number rs1801133) and SNP locus information were obtained from NCBI. TaqMan probes and primers were designed using Premier 6 software and Primer Express 3, respectively. TaqMan probes were labeled with FAM or HEX fluorescein. The probes and primers were synthesized by Shanghai Biotechnology Co., Ltd. The sequence of primers: 5'-ACCAACTTCCCAA CAGTCCA-3' and 5'-TGATGCCCTGAAAATCAAGC-3'; Probes for screening WT: HEX-5'-TTT GATGGAGGGAAA CAAACCTA-BHQ-X-3', and for *db* mutant FAM-5'-TT TTGATGGAGGTAACAAACCTA A-BHQ-X-3'. Genomic DNA was isolated from mouse tail clips. PCR reactions were performed on a Roche LC480 (Roche Diagnostics). Initial denaturation was 95°C for 1 min, then 40 cycles of 3 steps: 95°C for 15 s, 60°C for 40 s.

Body weight, food intake and blood glucose

Food intake, body weight and blood glucose were monitored monthly from 1 to 7 months. Blood glucose measurement and body weight were conducted between 15:00 and 16:00 h after 5 h of fasting. Blood glucose was sampled from tail tipping and measured on OneTouch Verio Flex™ meter (Johnson-Johnson). For HbA1c, the mice were anesthetized with isoflurane, blood was collected from the femoral artery, HbA1c, was measured at the 7th month by latex agglutination test using the automatic biochemical analyzer (ADVIA 2400, Siemens, Germany).

Intraperitoneal glucose tolerance test (IPGTT), insulin tolerance test (ITT) and pyruvate tolerance test (PTT)

For the IPGTT, all mice were fasted for 16 h with free access to water before intraperitoneal injection of a glucose solution (0.5 mg/kg of body wt) from 1 to 7

months. At the 7th month, all mice were fasted for 6 h prior to intraperitoneally injection of human biosynthetic insulin (1 units/kg of body wt) or sodium pyruvate (1 g/kg of body wt), respectively. Blood was obtained from the tail via a tail tip excision, and blood glucose measured by the glucometer at designed time points.

Measurement of plasma and hepatic biochemistry

The plasma alanine aminotransferase (ALT), glutamic-oxaloacetic transaminase (Mansouri *et al.* 1997) activities, total superoxide dismutase (SOD) and triglycerides (TG), total cholesterol (CHO), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and non-esterified free fatty acid (NEFA) concentrations were measured using commercial kits following the manufacturer's instructions (Jian-Cheng Bioengineering Incorporated Company Ltd. Nan Jing, China). Levels of ATP of the hepatic samples were measured using assay kits (Beyotime Bioengineering Incorporated Company Ltd. Shanghai, China) according to manufacturer's instructions. Total liver lipids were extracted following Folch's method (Folch *et al.* 1957). Liver TG and CHO concentrations were determined using the same kits used for serum samples.

Plasma and hepatic mitochondrial oxidative stress

Liver samples were flash-frozen and ground in liquid nitrogen. Ground tissue was homogenized on ice in PBS pH 7.4. The hepatic mitochondria were separated for the homogenate using differential centrifugation. This method was performed according to the protocol described by Rogers *et al.* (2011). Briefly, 500 mg liver tissue was washed and minced in PBS supplemented with 10 mM EDTA at 4°C . The tissue was homogenized with ten strokes with a glass homogenizer at 800 g in PBS buffer (pH 7.2) at 4°C , supplemented with PMSF and protease inhibitor cocktail (Thermo Fisher Scientific). The cell debris were removed by centrifugation at 1000 g for 5 min. Mitochondria were then isolated from the supernatant by centrifugation at 11,000 g for 10 min at 4°C . The final mitochondrial pellet was washed in MSHEB buffer and after another similar centrifugation step, was resuspended in a minimal volume of PBS buffer.

The levels of superoxide dismutase 2 (SOD2), glutathione (GSH), malondialdehyde (MDA) and total antioxidation capacity (T-AOC) in the mitochondria and plasma were measured using commercial kits following manufacturer's instructions (Cell Biolabs, Inc., San Diego, CA, USA). Protein content was determined using the BCA method

(Thermo Scientific). Enzyme activity units (nmol/mL) were normalized to protein concentration.

Liver ROS examination

The content of ROS in liver homogenate was determined by DCF fluorescence. The liver homogenate was diluted 100 times with PBS to make the cell suspension. The contents of ROS in different groups were measured by using the DCFH-DA detection kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instruction. The cells were incubated with DCFH-DA in the dark for 30 min at 37°C. After washed, Fluorescence intensity of the liver was detected at the condition of 488 nm excitation wavelength, 525 nm emission wavelength by using an enzyme labeling instrument (EnSpire Multimode Plate Reader, Perkin Elmer).

Serum cytokine measurement

Serum levels of IL1 β , IL6, TNF α and monocyte chemoattractant protein-1 (MCP1) concentrations were determined using ELISA kits (Beijing Dingguo Biotechnology Co. Ltd, Beijing, China). Each assay was carried out in duplicate. Absorbance was measured at 450 nm by means of a spectrophotometer (EnSpire Multimode Plate Reader, Perkin Elmer).

Liver histologic examination

The samples from the mouse liver were collected and fixed in 4% paraformaldehyde for 24 h. Fixed tissue was embedded in paraffin. Sections (4 μ m in thickness) were cut and processed by graded-alcohol dehydration, cleared in xylene, mounted and stained with H&E, and Masson Trichrome to evaluate the extent of hepatosteatosis, inflammation and fibrosis.

The degree of hepatosteatosis was semi-quantitatively evaluated using the scoring system recommended by the Pathology Committee of the Nonalcoholic Steatohepatitis Clinical Research Network (NSCRN) (Brunt *et al.* 1999, Franzen *et al.* 2005). Sixteen slides of mouse livers covering the whole spectrum of NAFLD, were blindly evaluated by an experienced animal pathologist in a blinded fashion.

The scoring system included semi-quantitative evaluation of three parameters: area of steatosis (0 $\leq$ 5%, 1 = 5–33%, 2 = 33–66%, 3 $\geq$ 66%), distribution of steatosis (0 = zone 3, 1 = zone 1, 2 = azonal, 3 = panacinar), and the presence/absence of microvesicular steatosis (0 = absent,

1 = present) (Kleiner *et al.* 2005). The difference between macrovesicular and microvesicular steatosis was defined by whether the vacuoles displaced the nucleus to the side (macrovesicular) or not (microvesicular).

Transmission electron microscopy (TEM)

After sacrificing the mice, livers were immediately cut into small pieces, fixed in PBS-buffered (pH 7.4) 2.5% glutaraldehyde for 4 h at room temperature (RT), and post-fixed in 1% buffered osmium tetroxide for 1 h at RT, dehydrated in a series of graded ethanol solution. The tissues were embedded in Epon 812 resin and placed in a 70°C oven for 72 h. The resin blocks were thick-sectioned, stained with 1% Toluidine blue and examined by light microscopy. One block each from Zone 1 (periportal) and Zone 3 (centrilobular) of the liver was chosen for each animal. The chosen blocks were ultrathin-sectioned (70 nm), placed on formvar-coated copper grids and stained with uranyl acetate and lead citrate. The grids were examined on a Hitachi H-7500 transmission electron microscope. For measurement of mitochondrial size, total of 150 mitochondria were randomly selected from 10 micrographs at 25,000 $\times$ magnification per group. Quantitative morphometric analysis using NIH Imaging Software was performed by a histologist blinded to the sample genotypes.

Western blotting analysis

Total proteins were extracted from the hepatic tissue or their mitochondria. Protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories). Equal amounts of total protein were resolved by 10–12% SD-PAGE and transferred to a polyvinylidene difluoride membrane. They were then incubated with the following primary antibodies: LIAS (1:1000, Proteintech, USA), SREBP1 (1:1000, Cell Signaling), FAS (1:1000, Cell Signaling), PGC1 α (1:800, Affinity Biosciences, Cincinnati, OH, USA), for 2 h. The membranes were subsequently washed in TBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:3000, Earthox, San Francisco, CA, USA) for 1 h at room temperature. Immunoreactive bands were visualized using the Amersham ECL Prime Western Blotting Detection Reagent with enhanced chemiluminescence (GE Healthcare Bio-Sciences) and exposed to ECL Hyperfilm (GE Healthcare Bio-Sciences). Membranes were incubated with rabbit polyclonal

Table 1 Gene name and GenBank accession number used in quantitative reverse transcriptase PCR.

Gene	Accession No.	Gene name	Function	Primer sequence
<i>Ucp2</i>	NM-011671.5	Uncoupling protein 2	Energy expenditure	5'-CTCTACGACTCTGTCAAACAGT-3' 5'-GGACCTTTACCACATCTGTAGG-3'
<i>Acads</i>	NM-007383.3	Acyl-Coenzyme A dehydrogenase, short chain	Mitochondrial β -oxidation	5'-AGTTCAAGCTGGCAGACATGGC-3' 5'-CCTGGTGGCTAATGGCGGTTG-3'
<i>Cpt1a</i>	NM-013495.2	Carnitine palmitoyltransferase 1a	Mitochondrial β -oxidation	5'-CTACATCACCCCAACCCATATT-3' 5'-GATCCCAGAAGACGAATAGGTT-3'
<i>Tfam</i>	NM-009360.4	Mitochondrial transcription factor A	Mitochondrial biogenesis	5'-GTGAGCAAGTATAAAGAGCAGC-3' 5'-CTGAACGAGGTCTTTTGGTTT-3'
<i>Sirt1</i>	NM-01159589.2	Sirtuin 1	Insulin sensitivity	5'-CGCTGTGGCAGATTGTTATTAA-3' 5'-TTGATCTGAAGTCAGGAATCCC-3'
<i>Prkaa2</i>	NM-01356568.1	protein kinase, AMP-activated, alpha 2 catalytic subunit	Regulating cellular energy	5'-CGATGAGGCTGTGAAGGAAGTCTG-3' 5'-GGAGGACTAGAGGCGAGGTAGAAC-3'

anti-GAPDH antibodies (1:5000, Proteintech) or anti-COXIV antibodies (1:5000, Proteintech) to serve as controls for equal loading. All Western blot analyses were performed at least three times. The density of proteins in each band was determined using image J2X software and expressed as relative to the density of the corresponding band of the control protein immunoblot.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from *Lias^{High/High}Lepr^{db/db}* and *Lias^{+/+}Lepr^{db/db}* liver using Trizol reagent (Thermo Fisher Scientific) and according to the manufacturer's instructions. RNA concentrations were determined by Nanodrop 1000 (Thermo Fisher Scientific). Five micrograms of RNA were reverse-transcribed to cDNA using the M-MLV kit (Thermo Fisher Scientific) following the instructions. The primer sequences used for the amplification of the indicated genes are listed in Table 1. Amplification and detection of specific products were performed using the LightCycler® 96 (Roche Diagnostics). All the samples were analyzed in duplicate. Cq values were generated by the Roche software. Non-obese *Lias^{+/+}Lepr^{db/+}* mice as control. Fold changes were calculated by the $2^{-\Delta\Delta Cq}$ method. β -actin was used as housekeeping gene for mRNA.

Quantification of mitochondrial DNA content

Mitochondrial DNA (mtDNA) content was quantified by real-time fluorescent quantitative PCR. Liver tissue from *Lias^{High/High}Lepr^{db/db}* and *Lias^{+/+}Lepr^{db/db}* mice was grinded, then added nucleic acid extract solution for the extraction of DNA. Mouse nuclear 18S rRNA was used as the internal control. The ratio of the expression of the target gene was got. The primers for detecting the cytochrome b gene of the murine mitochondrial genome were from the GenBank nucleotide sequences.

Statistical analysis

Data are expressed as the means $\pm$ S.E.M. Statistical analysis was performed with one-way ANOVA. $P < 0.05$ values were considered to be statistically significant. Effects of the dynamic changes of body weight and food consumption were assessed at various time points by multiple ANOVA for each mouse.

Results

Please note that all outliers have been included, which may be the result of inherent data variability.

Characterizations of *Lias^{High/High}Lepr^{db/db}* and *Lias^{+/+}Lepr^{db/db}* mice

Figure 1 reveals that our genotyping approach clearly identified *Lepr^{db/db}* mouse genotypes for monitoring characterizations of *Lepr^{db/+}* mice and *Lepr^{db/db}* mice. Figure 2A shows that *Lias^{High/High}Lepr^{db/db}* mice displayed significantly higher body weights, compared with *Lias^{+/+}Lepr^{db/db}* counterparts since the age of 2 months. Interestingly, after 3 months, the amount of food intake in male *Lias^{High/High}Lepr^{db/db}* was less than male *Lias^{+/+}Lepr^{db/db}* mice (Fig. 2B), but their net body weight gain was higher than *Lias^{+/+}Lepr^{db/db}* mice at 2 and 4 months (Fig. 2C). Females showed a similar tendency (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article). The data suggest that *Lias^{High/High}Lepr^{db/db}* mice utilized food for energy production more efficiently.

Changes of blood carbohydrate and lipid profiles

To investigate potential liver protection by overexpression of *Lias* gene, we examined dynamic changes of plasma

Figure 1

Identification of the *Lepr^{db/db}* mouse genotype. The Endpoint Fluorescent Scatter Plot shows allele distribution for identification of *Lepr^{db/db}* mouse genotype. Abscissa indicates FAM signal intensity value and ordinate represents HEX signal intensity value; Blue triangles, WT; Green, homozygote, and Red, heterozygote.

TG, CHO and glucose concentrations in the mice. Both male (Fig. 2D) and female (Supplementary Fig. 1C) *Lias^{High/High}Lepr^{db/db}* mice had significantly lower serum levels of TG than *Lias^{+/+}Lepr^{db/db}* mice after 3 months of age, especially female mice. Plasma CHO concentration in *Lias^{High/High}Lepr^{db/db}* mice started to increase more than *Lias^{+/+}Lepr^{db/db}* mice in both genders after 3 months. The divergence became larger with aging until the mice were sacrificed at 7 months of age (Fig. 2E and Supplementary Fig. 1D). However, plasma LDL levels did not significantly increase, and importantly, HDL concentration significantly increased in *Lias^{High/High}Lepr^{db/db}* mice (Table 2). The elevated levels of HDL would lead to more cholesterol from the blood and peripheral tissue to the liver for clearance. HDL may be boosted by the elevated endogenous LA since mounting evidence indicated that HDL itself was also an antioxidant (deGoma *et al.* 2008). In addition, *Lias^{High/High}Lepr^{db/db}* mice had decreased levels of serum NEFA and liver TG compared with *Lias^{+/+}Lepr^{db/db}* counterpart at 7 months of age (Fig. 2M and O). *Lias^{High/High}Lepr^{db/db}* mice had increased of liver weight compared with *Lias^{+/+}Lepr^{db/db}* counterpart at 7 months of age (Fig. 2N). Taken together, these data suggested that enhanced antioxidant content could mitigate lipid disorders.

Liver insulin resistant can result in glucose overproduction (Yki-Jarvinen 2014). Thus, we investigated impact of strengthened endogenous antioxidant reservoir on glucose metabolism and insulin resistance during NAFLD. Plasma glucose level became significantly lower in *Lias^{High/High}Lepr^{db/db}* mice compared

with those in *Lias^{+/+}Lepr^{db/db}* mice after 4 months of age (Fig. 2F and Supplementary Fig. 1B). Because HbA_{1c} remains significantly and independently associated with steatosis (Cosma *et al.* 2015), we measured plasma HbA_{1c} concentration. The data showed that HbA_{1c} level was significantly decreased at 7 months of age in *Lias^{High/High}Lepr^{db/db}* mice compared to *Lias^{+/+}Lepr^{db/db}* mice (Fig. 2K). Next, we performed IPGTT. As expected, male *Lias^{High/High}Lepr^{db/db}* mice cleared glucose more quickly than *Lias^{+/+}Lepr^{db/db}* mice during IPGTT from 1 to 7 months (Fig. 2G, H and Supplementary Fig. 2), suggesting that bolstered endogenous antioxidant capacity abated peripheral insulin resistance. To distinguish whether the decreased insulin resistance in *Lias^{High/High}Lepr^{db/db}* mice was resulted from increased absolute insulin production, we measured serum insulin in the male mice. Our results revealed that insulin value was significantly declined in 7-month-old *Lias^{High/High}Lepr^{db/db}* mice than their counterparts (Fig. 2L). At the 7th month, insulin resistance was examined using the ITT method. The results showed a significant reduction in blood glucose levels in *Lias^{High/High}Lepr^{db/db}* mice compared to the *Lias^{+/+}Lepr^{db/db}* mice (Fig. 2J). Furthermore, we observed a profound effect on the tolerance of both *Lias^{+/+}Lepr^{db/db}* and *Lias^{High/High}Lepr^{db/db}* mice to a challenge of pyruvate, a major substrate for hepatic gluconeogenesis, as *Lias^{High/High}Lepr^{db/db}* mice displayed significantly reduced plasma glucose levels following pyruvate challenge (Fig. 2I). The data clearly indicated that hyperinsulinemia resulting from insulin resistance diminished in *Lias^{High/High}Lepr^{db/db}* mice compared to *Lias^{+/+}Lepr^{db/db}* mice.

Plasma and liver mitochondria oxidative stress

To evaluate hepatic oxidative stress and antioxidant defense status, we measured the SOD2, T-AOC and MDA in plasma (Table 2), and these parameters and GSH in liver mitochondria (Table 3). The results indicated that significantly higher antioxidant/lower oxidative stress levels were observed in *Lias^{High/High}Lepr^{db/db}* compared to *Lias^{+/+}Lepr^{db/db}* mice. Mitochondrial abnormalities leading to decreased ATP production were observed in NASH patients (Caldwell *et al.* 1999, Cortez-Pinto *et al.* 1999a,b) and animals (Serviddio *et al.* 2008). To explore whether elevated endogenous antioxidant reservoir could protect liver mitochondria and thus retain energy homeostasis, we examined ATP yield in liver mitochondria isolated from males. Our result revealed that ATP production was significantly higher in *Lias^{High/High}Lepr^{db/db}* than *Lias^{+/+}Lepr^{db/db}* mice (Fig. 2Q). Collectively, these results

Figure 2

Body weight, food consumption, plasma lipid and glucose levels in *Lias*^{High/High}*Lepr*^{db/db} mice (close symbols) and *Lias*^{+/+}*Lepr*^{db/db} mice (open symbols). The body weight profile in (A) body weight, (B) the amount of food intake, and (C) the net gain of body weight in male mice. Dynamical changes of plasma triglyceride levels (D), and cholesterol concentrations (E), and plasma glucose levels (F), and intraperitoneal glucose tolerance test (IPGTT) (G) at 5 months of age and (H) at 6 months of age, and insulin tolerance test (ITT) (I) and pyruvate tolerance test (J) at 7 months of age. Plasma HbA1c concentrations (K), insulin concentrations (L), NEFA concentrations (M) at 7 months of age. Liver weight (N), liver triglyceride levels (O), liver ROS levels (P), liver mitochondrial ATP levels (Q) at 7 months old. Data are the means $\pm$ S.E.M. **P* < 0.05; ***P* < 0.01. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0606>.

Table 2 Biochemical parameters in *Lias*^{High/High}*Lepr*^{db/db} and *Lias*^{+/+}*Lepr*^{db/db} mice.

	<i>Lias</i> ^{+/+} <i>Lepr</i> ^{db/db}	<i>Lias</i> ^{High/High} <i>Lepr</i> ^{db/db}	<i>P</i> value
LDL (mmol/L)	2.34 $\pm$ 1.48	3.41 $\pm$ 1.42	0.118
HDL (mmol/L)	5.02 $\pm$ 1.81	7.69 $\pm$ 1.33	0.001
ALT (U/L)	53.01 $\pm$ 18.94	34.48 $\pm$ 10.52	0.042
GOT (U/L)	28.94 $\pm$ 7.46	18.91 $\pm$ 3.14	0.053
SOD (U/mL)	64.56 $\pm$ 10.97	72.71 $\pm$ 8.30	0.047
MDA (nmol/L)	13.57 $\pm$ 3.93	9.99 $\pm$ 2.86	0.040
T-AOC (μ M CRE/mL)	504.56 $\pm$ 6.66	556.55 $\pm$ 6.84	0.001
IL1 β (ng/L)	146.51 $\pm$ 43.67	84.15 $\pm$ 25.31	0.001
IL6 (ng/L)	68.45 $\pm$ 21.60	58.37 $\pm$ 20.74	0.301
TNF α (ng/L)	1691.92 $\pm$ 65.86	1104.37 $\pm$ 69.01	0.018
MCP1 (ng/L)	232.18 $\pm$ 6.69	159.29 $\pm$ 7.92	0.003

All mice were male at 7 months of age. Data are represented as mean $\pm$ S.E.M. (*n* = 10). Bold indicates *P* < 0.05.

Table 3 Liver mitochondria oxidative stress in *Lias^{High/High}Lepr^{db/db}* and *Lias^{+/+}Lepr^{db/db}* mice.

	<i>Lias^{+/+}Lepr^{db/db}</i>	<i>Lias^{High/High}Lepr^{db/db}</i>	P value
SOD2 (U/mgprot)	24.09 ± 2.90	30.94 ± 1.72	0.048
GSH (μg/mgprot)	196.11 ± 6.12	268.92 ± 6.83	0.001
MDA (nmol/mgprot)	5.26 ± 0.77	0.92 ± 0.31	0.001
T-AOC (mM CRE/mgprot)	4.89 ± 0.15	5.74 ± 0.16	0.001

All mice were male at 7 months of age. Data are represented as mean ± S.E.M. (*n* = 10). Bold indicates *P* < 0.05.

suggested that energy homeostasis was retained in *Lias^{High/High}Lepr^{db/db}* mice during NAFLD likely due to mitochondrial protection by endogenous antioxidants.

High endogenous antioxidant level attenuates inflammatory response

To determine whether the mice with enhanced antioxidant ability could reduce inflammatory responses, we measured blood pro-inflammatory cytokine levels. Compared with *Lias^{+/+}Lepr^{db/db}* mice, IL1β and TNFα levels in *Lias^{High/High}Lepr^{db/db}* mice were found to be statistically

lower (*P* < 0.05) (Table 3). However, statistically, no remarkable difference was seen in serum IL6 level between the two groups (Table 3). The results implied that oxidative stress promotes inflammation in liver steatosis.

Two distinct liver steatosis patterns in the different strains

Our data showed that both strains of the mice developed NAFLD (Fig. 3A, B, C and D). The steatosis area in the two strains was semi-quantitatively evaluated according to NSCRN scoring criteria. The total score in *Lias^{High/High}Lepr^{db/db}*

Figure 3

Histological patterns of liver steatosis. H&E staining of representative liver sections in *Lias^{+/+}Lepr^{db/db}* mice (A and C) and *Lias^{High/High}Lepr^{db/db}* mice (B and D) at 7-month of age. Original magnification 100× or 400×. A black arrow indicates areas of macrovesicular and a blue arrowhead points microvesicular steatosis. (E) NAFLD score. (F) Semi-quantitative lipid accumulation degrees and relative abundance of microvesicular and macrovesicular steatosis in *Lias^{High/High}Lepr^{db/db}* and *Lias^{+/+}Lepr^{db/db}* mice. Values represent means ± S.E.M. ***P* < 0.01.

Figure 4

Representative ultrastructural images of mitochondria. Enlarged mitochondria with irregularly shape (white arrows) in *Lias*^{+/+}*Lepr*^{db/db} mice (A). Mitochondria were relatively uniform in size and shape, and enlarged mitochondria were rare in *Lias*^{High/High}*Lepr*^{db/db} mice (B). The average size of mitochondria from six samples per group is presented (C). Levels of mtDNA were measured by real-time fluorescence quantitative PCR (D). Values represent means $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0606>.

mice was lower in *Lias*^{+/+}*Lepr*^{db/db} mice (Fig. 3E). In addition, the percentage of total steatosis area over entire liver area was slightly higher in *Lias*^{High/High}*Lepr*^{db/db} mice than in *Lias*^{+/+}*Lepr*^{db/db} mice (Fig. 3F). Interestingly, the lipid vesicle size and pattern of steatosis were obviously distinct between the two strains. *Lias*^{High/High}*Lepr*^{db/db} mice predominantly displayed macrovesicular steatosis characterized by a single, large vacuole of fat occupying the cytoplasm and displacing the nucleus to the cell periphery (Fig. 3B and D). On the contrary, *Lias*^{+/+}*Lepr*^{db/db} mouse liver principally exhibited microvesicular steatosis, featured by tiny lipid vesicles leaving the nucleus in the center of the cell and giving the hepatocyte a foamy, spongiocytic appearance (Fig. 3A and C). After semi-quantitatively comparing percentage of microvesicular region/entire steatosis region or macrovesicular region/entire steatosis region, the percentage of two types of steatosis in *Lias*^{High/High}*Lepr*^{db/db} and *Lias*^{+/+}*Lepr*^{db/db} mice was significantly different ($P < 0.01$) (Fig. 3F). In addition, we found that mitochondrial pleomorphism with increased variability in mitochondrial size and irregular enlargement of mitochondria in *Lias*^{+/+}*Lepr*^{db/db} mice (Fig. 4A). But enlarged mitochondria were rare and the mitochondria were rendered smaller and more

uniform in *Lias*^{High/High}*Lepr*^{db/db} mice (Fig. 4B and C). To determine whether the increasing of ATP activity in *Lias*^{High/High}*Lepr*^{db/db} mice (Fig. 2Q) was associated with mitochondrial biogenesis, the copy number of mtDNA, cytochrome b was measured by real-time PCR. Levels of mtDNA was significantly higher in *Lias*^{High/High}*Lepr*^{db/db} than *Lias*^{+/+}*Lepr*^{db/db} mice (Fig. 4D). These data suggest that high endogenous antioxidant capacity protects mitochondria during NAFLD.

Increased endogenous antioxidant activity modulates the gene expression of mitochondrial β -oxidation and bioenergetics

In order to further explore the underlying mechanisms of mitochondrial protection by increased endogenous antioxidant capacity during NAFLD, the expression of several key protein and genes associated with mitochondrial β -oxidation and bioenergetics was evaluated.

Our results showed that NAFLD markedly decreased LIAS protein expression ($P < 0.05$) in liver mitochondrion of *Lias*^{+/+}*Lepr*^{db/db} mice, overexpression of *Lias* gene of *Lias*^{High/High}*Lepr*^{db/db} mice was able to completely offset

Figure 5

Altered proteins and mRNAs expression levels. The gene expression was measured by Western blotting and qRT-PCR in the liver tissue or liver mitochondria from *Lias*^{High/High}*Lepr*^{db/db} mice versus *Lias*^{+/+}*Lepr*^{db/db} mice as controls. Expression of LIAS protein levels in mice liver mitochondria (A), SREBP1 protein levels (B), FAS protein levels (C), and PGC-1α protein levels (D) in mice liver, measured by Western blotting. COX IV or GAPDH served as an internal control for Western blotting. The relative expression of proteins was quantified using Image J Software. (E) q-PCR analysis of *Prkaa2*, *Acads*, *Cpt1a*, *Ucp2*, *Tfam* and *Sirt1* mRNA levels in liver tissues. β-actin served as an internal control for q-PCR. Results were expressed as mean ± s.e.m. and were compared by one-way ANOVA. **P* < 0.05; ***P* < 0.01.

these effects ($P < 0.01$) (Fig. 5A), and it is further verified that *Lias*^{High/High} mice have high endogenous antioxidant capacity. The SREBP1, FAS and PGC1α protein levels in liver of *Lias*^{+/+}*Lepr*^{db/db} mice were all significantly up-regulated compared to *Lias*^{+/+}*Lepr*^{db/+} mice as the control, overexpression of *Lias* gene in *Lias*^{High/High}*Lepr*^{db/db} mice was able to offset these effects ($P < 0.05$), suggesting that increased endogenous antioxidant capacity inhibited the steatosis of mice hepatocytes, inhibited the liver lipid metabolism disorder by NAFLD (Fig. 5B, C and D).

The results showed that NAFLD markedly decreased *Cpt1a* ($P < 0.05$) and *Tfam* gene expression ($P < 0.05$) in *Lias*^{+/+}*Lepr*^{db/db} mice, overexpression of *Lias* gene in *Lias*^{High/High}*Lepr*^{db/db} mice was able to completely offset these effects (Fig. 5E), suggesting that increased endogenous antioxidant capacity promoted mitochondrial β-oxidation

and biogenesis. The gene expression of *Prkaa2* in *Lias*^{+/+}*Lepr*^{db/db} mice was reduced by 75% compared to *Lias*^{+/+}*Lepr*^{db/+} mice as the control, whereas it was increased by 93% in *Lias*^{High/High}*Lepr*^{db/db} mice compared to *Lias*^{+/+}*Lepr*^{db/db} mice (Fig. 5E). The protein encoded by *Prkaa2* gene is a catalytic subunit of the AMP-activated protein kinase (AMPK). The data suggested that NAFLD was associated with inhibited AMPK but *Lias* gene overexpression could partially reverse the activity. Compared with the control, there was a non-significant decrease ($P = 0.24$) in mRNA expression of *Ucp2* (−15%) in *Lias*^{+/+}*Lepr*^{db/db} mice, but this decrease was significantly reversed in *Lias*^{High/High}*Lepr*^{db/db} mice ($P < 0.01$) (Fig. 5E). *Sirt1* gene expression was decreased in *Lias*^{+/+}*Lepr*^{db/db} mouse liver compared to the control and its expression further reduced in *Lias*^{High/High}*Lepr*^{db/db} mice (Fig. 5E).

Discussion

In this study, we intensively investigated the novel mouse model according to criteria for NAFLD patients (Santhekadur *et al.* 2018). We found that this model resembled many features of human NAFLD, especially for the contribution of mitochondrial dysfunction resulting from oxidative stress to the diseases. Our data clearly demonstrated that oxidative stress played a critical role in the development of NAFLD and that enhanced endogenous antioxidant reservoir significantly attenuated NAFLD in *Lias^{High/High}Lepr^{db/db}* mice.

We evaluated hepatic oxidative stress in the mouse model by measuring the multiple antioxidant/oxidative stress biomarkers in plasma and/or liver mitochondria. All of these results indicated that the oxidative stress level was significantly lower and the antioxidant content was significantly higher in *Lias^{High/High}Lepr^{db/db}* mice than *Lias^{+/+}Lepr^{db/db}* mice. Thus, our results provide solid evidence to support the role of oxidative stress in the development of NAFLD.

Growing evidence suggests a pivotal role of mitochondrial dysfunction in the development of NASH (Koliaki *et al.* 2015). We observed that as a consequence of increased mitochondrial antioxidant content, *Lias^{High/High}Lepr^{db/db}* mice exhibited milder liver damage. Interestingly, the lipid vesicle size and pattern of steatosis between the two strains were noticeably different. Macrovesicular steatosis is a common form of NAFLD. Unlike microvesicular steatosis, macrovesicular steatosis does not consistently correlate with features of cell injury and steatohepatitis since it rarely progresses to fibrosis or cirrhosis (Contos *et al.* 2004, Oleszczuk *et al.* 2007, Tandra *et al.* 2011). In contrast, the presence of microvesicular steatosis in humans is significantly associated with histological hallmarks of cellular injury (Sherlock 1995), as a result of mitochondrial dysfunction with subsequent impaired β -oxidation giving rise to liver triglyceride accumulation (Fromenty *et al.* 1997). In microvesicular steatosis, NEFA is poorly oxidized in mitochondria, resulting in a considerable increase in liver and plasma (Pessayre *et al.* 1999, Bjorndal *et al.* 2018). These observations were supported by our finding that plasma NEFA level was significantly lower in *Lias^{High/High}Lepr^{db/db}* mice. Moreover, megamitochondria formation reflects hepatic injury to the mitochondrial electron transport chain in patients (Le *et al.* 2004), but corresponding ultrastructural change in rodents is mitochondrial swelling (Kirsch *et al.* 2003), which occurred in *Lias^{+/+}Lepr^{db/db}* mice. The microvesicular steatosis, co-presence with enlarged

mitochondria, may serve as additional histological evidence for mitochondrial dysfunction.

Inflammation is another important factor in mediating the pathogenesis of NAFLD. Elevated levels of pro-inflammatory cytokines and chemokines including IL1 β , TNF α and MCP1 are found in the different aspects of pathophysiology of human NAFLD/NASH (Singal *et al.* 2011). They are significantly declined in *Lias^{High/High}Lepr^{db/db}* mice suggest that elevated endogenous antioxidant ability help suppress inflammation to curb the NAFLD development toward NASH. This is not unexpected since our previous study already demonstrated that LA was able to quench inflammation (Yi *et al.* 2009).

CPT1 α , AMPK and *Tfam* gene is downregulated in *Lias^{+/+}Lepr^{db/db}* mice, which partially or entirely offset in *Lias^{High/High}Lepr^{db/db}* mice. *CPT1 α* is an enzyme present in the outer mitochondria membrane that regulates β -oxidation by transferring long-chain fatty acids into the mitochondria (Schreurs *et al.* 2010). Therefore, we postulated that the anti-adipogenic effect of LA was partially associated with the expression of β -oxidation-related genes. AMPK plays an important role in regulating intracellular energy metabolism, and has been identified as a major regulator of mitochondrial biogenesis (Bergeron *et al.* 2001). The increase in hepatic *Tfam* mRNA level indicates that elevated endogenous antioxidant induces mitochondrial biogenesis, which may increase the capacities of mitochondrial respiration and lipid β -oxidation to support ATP production. Additionally, the hepatic mRNA levels of two genes encoding *Acads* and *Sirt1* were either unchanged or decreased in *Lias^{High/High}Lepr^{db/db}*, compared with *Lias^{+/+}Lepr^{db/db}* mice. The results of this study suggest that overexpression of *Lias* gene does not affect short-chain fatty acid β -oxidation.

Overall, our new model shares many pathological changes with NAFLD patients, especially oxidative stress-derived changes. Overexpression of *Lias* gene effectively reduces oxidative stress and protects mitochondria, and consequently attenuates NAFLD.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0606>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was funded by the National Natural Science Foundation of China (81703183 to Guangcui Xu and 81773399 to Yingzheng Zhao) and Scientific and Technological Research Project of Henan Provincial Science and Technology Department (182102310078 to Guangcui Xu).

Author contribution statement

Yingzheng Zhao and Xianwen Yi involved in the conceptualization of the study. Guangcui Xu, Tingting Yan, Qiang Peng and Haibin Li involved in methodology. Yingzheng Zhao and Weidong Wu contributed to data validation. Guangcui Xu, Tingting Yan, Qiang Peng and Yingzheng Zhao involved in data curation. Guangcui Xu and Yingzheng Zhao contributed to writing – original draft preparation. Xianwen Yi and Weidong Wu contributed to writing – review and editing. Yingzheng Zhao and Haibin Li involved in supervision. Weidong Wu contributed to project administration. Xianwen Yi contributed to manuscript submission. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors thank Xuxu Zhang and Xiaonan Zhai for participation in this study, especially for mouse colony maintenance.

References

- Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF & Shulman GI 2001 Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *American Journal of Physiology: Endocrinology and Metabolism* **281** E1340–E1346. (<https://doi.org/10.1152/ajpendo.2001.281.6.E1340>)
- Bjorndal B, Alteras EK, Lindquist C, Svardal A, Skorve J & Berge RK 2018 Associations between fatty acid oxidation, hepatic mitochondrial function, and plasma acylcarnitine levels in mice. *Nutrition and Metabolism* **15** 10. (<https://doi.org/10.1186/s12986-018-0241-7>)
- Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA & Bacon BR 1999 Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *American Journal of Gastroenterology* **94** 2467–2474. (<https://doi.org/10.1111/j.1572-0241.1999.01377.x>)
- Caldwell S & Lackner C 2017 Perspectives on NASH histology: cellular ballooning. *Annals of Hepatology* **16** 182–184. (<https://doi.org/10.5604/16652681.1231550>)
- Caldwell SH, Oelsner DH, Iezzoni JC, Hespenheide EE, Battle EH & Driscoll CJ 1999 Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. *Hepatology* **29** 664–669. (<https://doi.org/10.1002/hep.510290347>)
- Contos MJ, Choudhury J, Mills AS & Sanyal AJ 2004 The histologic spectrum of nonalcoholic fatty liver disease. *Clinics in Liver Disease* **8** 481–500, vii. (<https://doi.org/10.1016/j.cld.2004.04.013>)
- Cortez-Pinto H, Chatham J, Chacko VP, Arnold C, Rashid A & Diehl AM 1999a Alterations in liver ATP homeostasis in human nonalcoholic steatohepatitis: a pilot study. *JAMA* **282** 1659–1664. (<https://doi.org/10.1001/jama.282.17.1659>)
- Cortez-Pinto H, Camilo ME, Baptista A, De Oliveira AG & De Moura MC 1999b Non-alcoholic fatty liver: another feature of the metabolic syndrome? *Clinical Nutrition* **18** 353–358. ([https://doi.org/10.1016/s0261-5614\(99\)80015-6](https://doi.org/10.1016/s0261-5614(99)80015-6))
- Cosma A, Cecchet D, Gaiani S, Coracina A, Pellizzari P, Pizzi C, Veronese N, Sacerdoti D & Tessari P 2015 Clinical and biochemical determinants of the extent of liver steatosis in type 2 diabetes mellitus. *European Journal of Gastroenterology and Hepatology* **27** 1386–1391. (<https://doi.org/10.1097/MEG.0000000000000462>)
- Day CP & James OF 1998 Steatohepatitis: a tale of two ‘hits’? *Gastroenterology* **114** 842–845. ([https://doi.org/10.1016/s0016-5085\(98\)70599-2](https://doi.org/10.1016/s0016-5085(98)70599-2))
- deGoma EM, deGoma RL & Rader DJ 2008 Beyond high-density lipoprotein cholesterol levels evaluating high-density lipoprotein function as influenced by novel therapeutic approaches. *Journal of the American College of Cardiology* **51** 2199–2211. (<https://doi.org/10.1016/j.jacc.2008.03.016>)
- Dorsam B & Fahrer J 2016 The disulfide compound alpha-lipoic acid and its derivatives: a novel class of anticancer agents targeting mitochondria. *Cancer Letters* **371** 12–19. (<https://doi.org/10.1016/j.canlet.2015.11.019>)
- Filozof C, Goldstein BJ, Williams RN & Sanyal A 2015 Non-alcoholic steatohepatitis: limited available treatment options but promising drugs in development and recent progress towards a regulatory approval pathway. *Drugs* **75** 1373–1392. (<https://doi.org/10.1007/s40265-015-0437-3>)
- Folch J, Lees M & Sloane Stanley GH 1957 A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226** 497–509.
- Franzen LE, Ekstedt M, Kechagias S & Bodin L 2005 Semiquantitative evaluation overestimates the degree of steatosis in liver biopsies: a comparison to stereological point counting. *Modern Pathology* **18** 912–916. (<https://doi.org/10.1038/modpathol.3800370>)
- Fromenty B, Berson A & Pessayre D 1997 Microvesicular steatosis and steatohepatitis: role of mitochondrial dysfunction and lipid peroxidation. *Journal of Hepatology* **26** (Supplement 1) 13–22. ([https://doi.org/10.1016/s0168-8278\(97\)82328-8](https://doi.org/10.1016/s0168-8278(97)82328-8))
- Groenbaek K, Friis H, Hansen M, Ring-Larsen H & Krarup HB 2006 The effect of antioxidant supplementation on hepatitis C viral load, transaminases and oxidative status: a randomized trial among chronic hepatitis C virus-infected patients. *European Journal of Gastroenterology and Hepatology* **18** 985–989. (<https://doi.org/10.1097/01.meg.0000231746.76136.4a>)
- Ka SO, Bang IH, Bae EJ & Park BH 2017 Hepatocyte-specific sirtuin 6 deletion predisposes to nonalcoholic steatohepatitis by up-regulation of Bach1, an Nrf2 repressor. *FASEB Journal* **31** 3999–4010. (<https://doi.org/10.1096/fj.201700098RR>)
- Kirsch R, Clarkson V, Shephard EG, Marais DA, Jaffer MA, Woodburne VE, Kirsch RE & Hall PdeL 2003 Rodent nutritional model of non-alcoholic steatohepatitis: species, strain and sex difference studies. *Journal of Gastroenterology and Hepatology* **18** 1272–1282. (<https://doi.org/10.1046/j.1440-1746.2003.03198.x>)
- Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, *et al.* 2005 Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41** 1313–1321. (<https://doi.org/10.1002/hep.20701>)
- Koliaki C, Szendroedi J, Kaul K, Jelenik T, Nowotny P, Jankowiak F, Herder C, Carstensen M, Krausch M, Knoefel WT, *et al.* 2015 Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metabolism* **21** 739–746. (<https://doi.org/10.1016/j.cmet.2015.04.004>)
- Le TH, Caldwell SH, Redick JA, Sheppard BL, Davis CA, Arseneau KO, Iezzoni JC, Hespenheide EE, Al-Osaimi A & Peterson TC 2004 The zonal distribution of megamitochondria with crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* **39** 1423–1429. (<https://doi.org/10.1002/hep.20202>)
- Lindquist C, Bjorndal B, Rossmann CR, Svardal A, Hallstrom S & Berge RK 2018 A fatty acid analogue targeting mitochondria exerts a plasma triacylglycerol lowering effect in rats with impaired carnitine biosynthesis. *PLoS ONE* **13** e0194978. (<https://doi.org/10.1371/journal.pone.0194978>)

- Liu J 2008 The effects and mechanisms of mitochondrial nutrient alpha-lipoic acid on improving age-associated mitochondrial and cognitive dysfunction: an overview. *Neurochemical Research* **33** 194–203. (<https://doi.org/10.1007/s11064-007-9403-0>)
- Mansouri A, Gaou I, Fromenty B, Berson A, Letteron P, Degott C, Erlinger S & Pessayre D 1997 Premature oxidative aging of hepatic mitochondrial DNA in Wilson's disease. *Gastroenterology* **113** 599–605. (<https://doi.org/10.1053/gast.1997.v113.pm9247482>)
- Namazi N, Larijani B & Azadbakht L 2018 Alpha-lipoic acid supplement in obesity treatment: a systematic review and meta-analysis of clinical trials. *Clinical Nutrition* **37** 419–428. (<https://doi.org/10.1016/j.clnu.2017.06.002>)
- Oleszczuk A, Spannbaumer M, Tannapfel A, Bluher M, Hengstler J, Pietsch UC, Schuhmacher A, Wittkekind C, Hauss JP & Schon MR 2007 Regenerative capacity differs between micro- and macrovesicular hepatic steatosis. *Experimental and Toxicologic Pathology* **59** 205–213. (<https://doi.org/10.1016/j.etp.2007.05.009>)
- Pais R, Barritt AS, Calmus Y, Scatton O, Runge T, Lebray P, Poynard T, Ratzliff V & Conti F 2016 NAFLD and liver transplantation: current burden and expected challenges. *Journal of Hepatology* **65** 1245–1257. (<https://doi.org/10.1016/j.jhep.2016.07.033>)
- Pamuk GE & Sonsuz A 2003 N-acetylcysteine in the treatment of non-alcoholic steatohepatitis. *Journal of Gastroenterology and Hepatology* **18** 1220–1221. (<https://doi.org/10.1046/j.1440-1746.2003.03156.x>)
- Park KG, Min AK, Koh EH, Kim HS, Kim MO, Park HS, Kim YD, Yoon TS, Jang BK, Hwang JS, *et al.* 2008 Alpha-lipoic acid decreases hepatic lipogenesis through adenosine monophosphate-activated protein kinase (AMPK)-dependent and AMPK-independent pathways. *Hepatology* **48** 1477–1486. (<https://doi.org/10.1002/hep.22496>)
- Pessayre D, Mansouri A, Haouzi D & Fromenty B 1999 Hepatotoxicity due to mitochondrial dysfunction. *Cell Biology and Toxicology* **15** 367–373. (<https://doi.org/10.1023/a:1007649815992>)
- Rogers GW, Brand MD, Petrosyan S, Ashok D, Elorza AA, Ferrick DA & Murphy AN 2011 High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS ONE* **6** e21746. (<https://doi.org/10.1371/journal.pone.0021746>)
- Sahai A, Malladi P, Pan X, Paul R, Melin-Aldana H, Green RM & Whittington PF 2004 Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. *American Journal of Physiology: Gastrointestinal and Liver Physiology* **287** G1035–G1043. (<https://doi.org/10.1152/ajpgi.00199.2004>)
- Santhekadur PK, Kumar DP & Sanyal AJ 2018 Preclinical models of non-alcoholic fatty liver disease. *Journal of Hepatology* **68** 230–237. (<https://doi.org/10.1016/j.jhep.2017.10.031>)
- Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, Neuschwander-Tetri BA, Lavine JE, Tonascia J, Unalp A, *et al.* 2010 Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *New England Journal of Medicine* **362** 1675–1685. (<https://doi.org/10.1056/NEJMoa0907929>)
- Schreurs M, Kuipers F & van der Leij FR 2010 Regulatory enzymes of mitochondrial beta-oxidation as targets for treatment of the metabolic syndrome. *Obesity Reviews* **11** 380–388. (<https://doi.org/10.1111/j.1467-789X.2009.00642.x>)
- Serviddio G, Bellanti F, Tamborra R, Rollo T, Capitanio N, Romano AD, Sastre J, Vendemiale G & Altomare E 2008 Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. *Gut* **57** 957–965. (<https://doi.org/10.1136/gut.2007.147496>)
- Sherlock S 1995 Alcoholic liver disease. *Lancet* **345** 227–229. ([https://doi.org/10.1016/S0140-6736\(95\)90226-0](https://doi.org/10.1016/S0140-6736(95)90226-0))
- Singal AK, Jampala SC & Weinman SA 2011 Antioxidants as therapeutic agents for liver disease. *Liver International* **31** 1432–1448. (<https://doi.org/10.1111/j.1478-3231.2011.02604.x>)
- Solis-Munoz P, Solis-Herruzo JA, Fernandez-Moreira D, Gomez-Izquierdo E, Garcia-Consuegra I, Munoz-Yague T & Garcia Ruiz I 2011 Melatonin improves mitochondrial respiratory chain activity and liver morphology in ob/ob mice. *Journal of Pineal Research* **51** 113–123. (<https://doi.org/10.1111/j.1600-079X.2011.00868.x>)
- Takahashi Y 2012 Essential roles of growth hormone (GH) and insulin-like growth factor-I (IGF-I) in the liver. *Endocrine Journal* **59** 955–962. (<https://doi.org/10.1507/endocrj.ej12-0322>)
- Tanaka Y, Kaibori M, Miki H, Nakatake R, Tokuhara K, Nishizawa M, Okumura T & Kwon AH 2015 Alpha-lipoic acid exerts a liver-protective effect in acute liver injury rats. *Journal of Surgical Research* **193** 675–683. (<https://doi.org/10.1016/j.jss.2014.08.057>)
- Tandra S, Yeh MM, Brunt EM, Vuppalanchi R, Cummings OW, Unalp-Arida A, Wilson LA & Chalasani N 2011 Presence and significance of microvesicular steatosis in nonalcoholic fatty liver disease. *Journal of Hepatology* **55** 654–659. (<https://doi.org/10.1016/j.jhep.2010.11.021>)
- Vajro P, Mandato C, Franzese A, Ciccimarra E, Lucariello S, Savoia M, Capuano G & Migliaro F 2004 Vitamin E treatment in pediatric obesity-related liver disease: a randomized study. *Journal of Pediatric Gastroenterology and Nutrition* **38** 48–55. (<https://doi.org/10.1097/00005176-200401000-00012>)
- Wong VW & Wong GL 2016 A LEAN treatment for non-alcoholic steatohepatitis. *Lancet* **387** 628–630. ([https://doi.org/10.1016/S0140-6736\(15\)00982-4](https://doi.org/10.1016/S0140-6736(15)00982-4))
- Wortham M, He L, Gyamfi M, Copple BL & Wan YJ 2008 The transition from fatty liver to NASH associates with SAMe depletion in db/db mice fed a methionine choline-deficient diet. *Digestive Diseases and Sciences* **53** 2761–2774. (<https://doi.org/10.1007/s10620-007-0193-7>)
- Xu L, Hiller S, Simington S, Nickenleit V, Maeda N, James LR & Yi X 2016 Influence of different levels of lipoic acid synthase gene expression on diabetic nephropathy. *PLoS ONE* **11** e0163208. (<https://doi.org/10.1371/journal.pone.0163208>)
- Yi X & Maeda N 2005 Endogenous production of lipoic acid is essential for mouse development. *Molecular and Cellular Biology* **25** 8387–8392. (<https://doi.org/10.1128/MCB.25.18.8387-8392.2005>)
- Yi X & Maeda N 2006 Alpha-lipoic acid prevents the increase in atherosclerosis induced by diabetes in apolipoprotein E-deficient mice fed high-fat/low-cholesterol diet. *Diabetes* **55** 2238–2244. (<https://doi.org/10.2337/db06-0251>)
- Yi X, Kim K, Yuan W, Xu L, Kim HS, Homeister JW, Key NS & Maeda N 2009 Mice with heterozygous deficiency of lipoic acid synthase have an increased sensitivity to lipopolysaccharide-induced tissue injury. *Journal of Leukocyte Biology* **85** 146–153. (<https://doi.org/10.1189/jlb.0308161>)
- Yi X, Xu L, Hiller S, Kim HS & Maeda N 2012 Reduced alpha-lipoic acid synthase gene expression exacerbates atherosclerosis in diabetic apolipoprotein E-deficient mice. *Atherosclerosis* **223** 137–143. (<https://doi.org/10.1016/j.atherosclerosis.2012.04.025>)
- Yki-Jarvinen H 2014 Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet: Diabetes and Endocrinology* **2** 901–910. ([https://doi.org/10.1016/S2213-8587\(14\)70032-4](https://doi.org/10.1016/S2213-8587(14)70032-4))
- Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L & Wymers M 2016 Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64** 73–84. (<https://doi.org/10.1002/hep.28431>)

Received in final form 23 October 2020

Accepted 24 November 2020

Accepted Manuscript published online 1 December 2020