adolescents with high hepatic fat content show lower whole-body insulin sensitivity independent of visceral fat and intramyocellular lipid content. These articles indicate a strong correlation between fatty liver and muscle insulin resistance in humans, but it was still unknown whether fatty liver disease directly causes muscle insulin resistance in obesity. The liver is a major site for the production of bioactive secretory proteins called hepatokines (12,19). Many lines of evidence have reported that the dysregulation of the production of hepatokines such as SeP or fetuin A is involved in the development of systemic insulin resistance (12,13,46,47). This study demonstrates a previously unrecognized role for LECT2 in glucose metabolism and suggests that LECT2 is a strong candidate to explain a mechanism by which the fatty liver leads to whole-body insulin resistance in obesity.

The energy depletion–sensing kinase AMPK functions as a metabolic sensor that promotes insulin sensitivity (36). Exercise is known to increase the phosphorylation and activity of AMPK in skeletal muscle. Early reports have shown that exercise-induced AMPK phosphorylation also is observed in the liver (37,38). On the other hand, an HFD is reported to decrease AMPK phosphorylation in the liver, probably because of excessive accumulation of energy (48,49). Negative regulation of LECT2 by the energy depletion–sensing kinase AMPK supports the concept that LECT2 functions as a hepatokine that senses overnutrition. One limitation of this study is that the molecular mechanism by which AMPK reduces *Lect2* expression is still unknown. Additional studies are needed to determine the transcriptional factors that negatively regulate *Lect2* expression downstream of AMPK pathway.

JNK is a mitogen-activated protein kinase that is activated by various stimuli, including cytokines, reactive oxygen species, endoplasmic reticulum stress, and metabolic changes (41). JNK plays a major role in the development of insulin resistance induced by an HFD through phosphorylating insulin receptor substrates at specific serine and threonine residues (50,51). Several more recent studies suggest a role for JNK in the development of insulin resistance in skeletal muscle, as well as in the liver or adipose tissue. Ferreira et al. (52) reported an increase of JNK phosphorylation and a decrease of insulin-stimulated Akt phosphorylation in the skeletal muscle of patients with nonalcoholic steatohepatitis. Henstridge et al. (53) showed that muscle-specific overproduction of CA JNK induces muscle insulin resistance in mice. Conversely, Sabio et al. (54) revealed that muscle-specific JNK knockout mice exhibit improved insulin sensitivity in skeletal muscle. Hence the overproduction of LECT2 in the liver may contribute, at least in part, to JNK phosphorylation and the subsequent insulin resistance observed in the skeletal muscle of obese patients. However, the mechanism by which LECT2 increases JNK phosphorylation remains unresolved. Our results suggest that LECT2-induced JNK activation in cultured myocytes is independent of inflammation or endoplasmic reticulum stress (Supplementary Fig. 4). Identification of the LECT2 receptor and characterization of its downstream signaling will provide insights into the involvement of LECT2 in JNK phosphorylation.

We show that overexpression of *Lect2* does not alter the inflammatory response in cultured myotubes. Early reports suggest that *Lect2* exerts different effects on inflammation depending on various pathological conditions. Inflammation observed in autoimmune disorders such as collagen antibody–induced arthritis or concanavalin A–induced hepatitis is reported to be suppressed by *Lect2* (17,55). *Lect2* also attenuates β-catenin–induced inflammation associated with hepatocellular carcinoma in mouse models (18). On the other hand, a more recent report showed that *Lect2* activates lipopolysaccharide-stimulated macrophages via the CD209a receptor, resulting in an improvement in the prognosis for survival in mice with bacterial sepsis (56). Because we found no expression levels of *CD209a* in C2C12 myotubes in real-time PCR experiments (data not shown), *Lect2*-induced insulin resistance in cultured myocytes is likely to be independent of inflammatory response via the CD209a receptor. However, it is unknown whether *Lect2* affects macrophages observed in the adipose tissue of obesity. The actions of *Lect2* on low-grade inflammation seen in obesity are now under investigation.

Interestingly, although *Lect2* knockout mice showed an increase of insulin signaling in the skeletal muscle when fed an HFD or regular chow, this increase was abolished after 60 h of starvation. Serum levels of LECT2 were increased by an HFD (Fig. 2*E*), whereas they were decreased by starvation in wild-type mice (Fig. 5*C*). Hence it seems most likely that the difference in serum LECT2 levels between wild-type and knockout mice was enhanced by an HFD, whereas it was reduced by starvation. The abolishment of the insulin-sensitive phenotypes in *Lect2* knockout mice after starvation may be explained by reduction of the difference in serum LECT2 levels. These results suggest that *Lect2* plays a major role in the regulation of insulin sensitivity in overnutritional conditions, but not in the undernutritional ones.

body weight; 10 min after insulin administration, the hind-limb muscles were removed. *L*: Glucose infusion rate (GIR), endogenous glucose production (EGP), and rate of glucose disposal (Rd) during hyperinsulinemic-euglycemic clamp in *Lect2*-deficient and wild-type mice ($n = 6$ or 7). *M*: mRNA levels of genes involved in myogenesis and mitochondria in skeletal muscle of *Lect2*-deficient and wild-type mice ($n = 4$ or 5). Data in *A–I* and *K–M* represent the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (*Lect2*-deficient mice vs. wild-type mice).

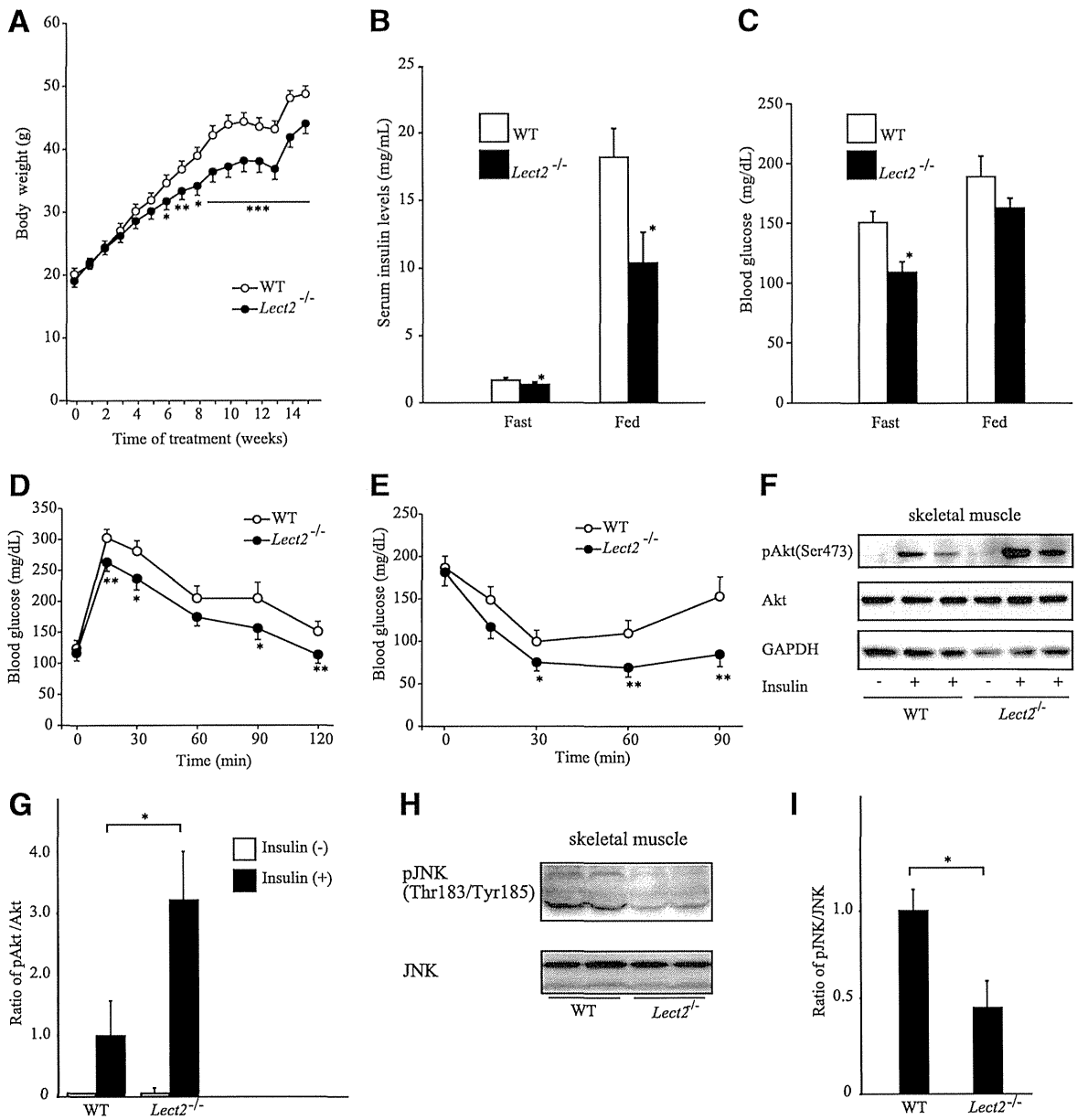

Figure 4—*Lect2* deletion attenuates muscle insulin resistance in diet-induced obesity in mice. *A*: Changes of body weight of *Lect2*-deficient and wild-type (WT) mice fed an HFD (n = 9–13). *B*: Serum insulin levels of *Lect2*-deficient and wild-type mice fed an HFD for 11 weeks in a fed condition and after 12 h of fasting. (n = 9–13). *C*: Blood glucose levels of *Lect2*-deficient and wild-type mice fed an HFD for 11 weeks in a fed condition and after 12 h of fasting (n = 9–13). *D*: Intraperitoneal glucose tolerance tests in *Lect2*-deficient and wild-type mice fed an HFD for 9 weeks (n = 9–13). Glucose was administered at doses of 0.5 g/kg body weight. *E*: Intraperitoneal insulin tolerance tests in *Lect2*-deficient and wild-type mice fed an HFD for 10 weeks (n = 9–13). Insulin was administered at doses of 1.2 units/kg body weight. *F* and *G*: Western blot analysis and quantification of phosphorylated Akt in skeletal muscle of *Lect2*-deficient and wild-type mice (n = 3 or 4), respectively. *H* and *I*: Western blot analysis and quantification of phosphorylated JNK in skeletal muscle of *Lect2*-deficient and wild-type mice (n = 3 or 4), respectively. Mice were stimulated with insulin (administered through the vena cava) at doses of 1 unit/kg body weight; 2 min after insulin administration, the hind-limb muscles were removed. Data in *A–E* and *G* represent the means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (*Lect2*-deficient mice vs. wild-type mice).

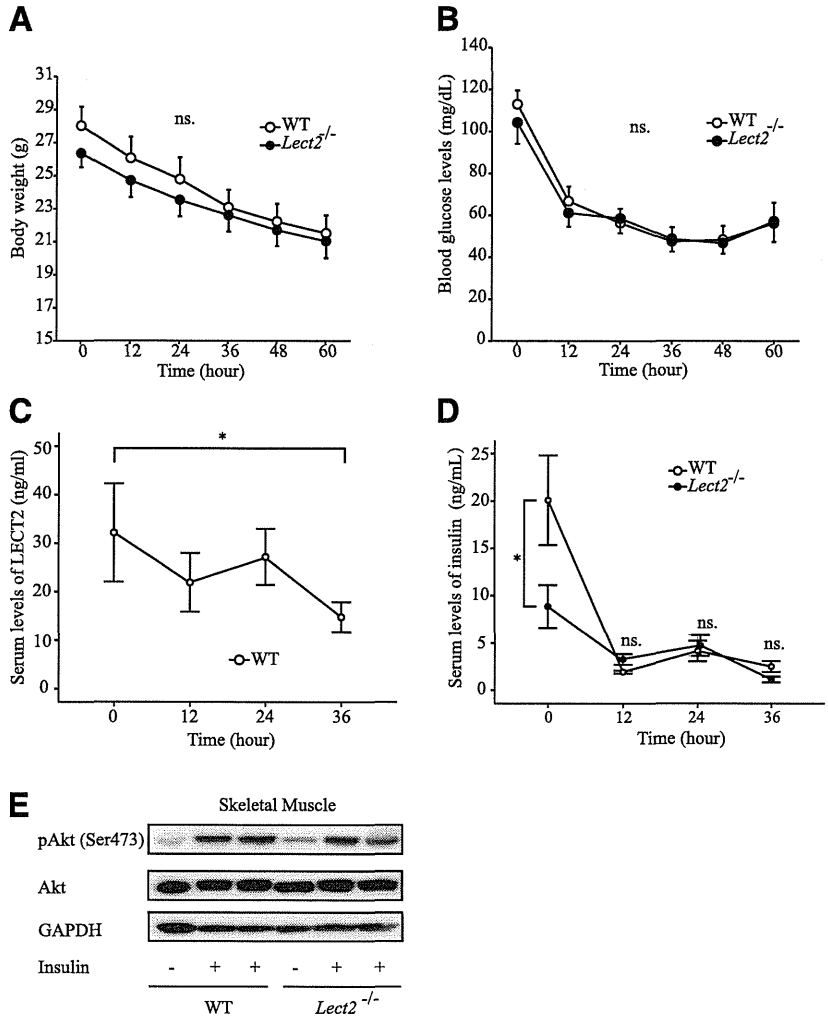

Figure 5—Starvation abolishes the insulin-sensitive phenotype in *Lect2*-deficient mice. *A*: Body weight of 20-week-old female *Lect2*-deficient and wild-type (WT) mice (n = 5) during starvation. *B*: Blood glucose levels of *Lect2*-deficient and wild-type mice (n = 5) during starvation. *C*: Serum levels of insulin of *Lect2*-deficient and wild-type mice (n = 4 or 5) in a fed condition or after starvation for 36 h (paired t test). *D*: Serum levels of LECT2 of wild-type mice (n = 4 or 5) in a fed condition or after starvation for 36 h. *E*: Western blot analysis of phosphorylated Akt in skeletal muscle of *Lect2*-deficient and wild-type mice after 60 h of starvation. Mice were stimulated with insulin (administered intraperitoneally); 15 min after insulin administration, mice were anesthetized and hind-limb muscle samples were removed for analysis. Data in *A–D* represent the means ± SEM. Data in *D* were assessed by paired t tests. *P < 0.05.

Our data reveal that a 60% HFD for 1 week resulted in a concurrent decrease of insulin signaling in skeletal muscle and an increase of circulating levels of LECT2 in C57BL/6J mice. A previous clinical report showed that overfeeding and inactivity for only 3 days impaired insulin sensitivity in healthy young men (57). Impairment of insulin sensitivity occurred before changes in body composition such as total fat mass and visceral fat area. However, additional clinical studies are required to determine whether eating an HFD for several days indeed induces simultaneous alterations of circulating LECT2 and muscle insulin sensitivity in humans.

C2C12 myocytes transfected with plasmid encoding LECT2 showed an impairment of myotube differentiation and insulin signal transduction (Fig. 6*C* and *D*). The presence of LECT2 protein in the culture medium (Fig. 6*B*) suggests that LECT2 derived from the pLect2 acted on the cells in an autocrine or paracrine manner. Because the half-life of LECT2 protein was predicted to be short because of the low molecular weight of LECT2 (16 kDa), we initially overexpressed *Lect2* in the cultured myocytes to examine the chronic actions of LECT2. In the next experiments, we directly treated well-differentiated C2C12 myotubes with recombinant LECT2 protein for 3 h (Fig. 6*F*) to exclude

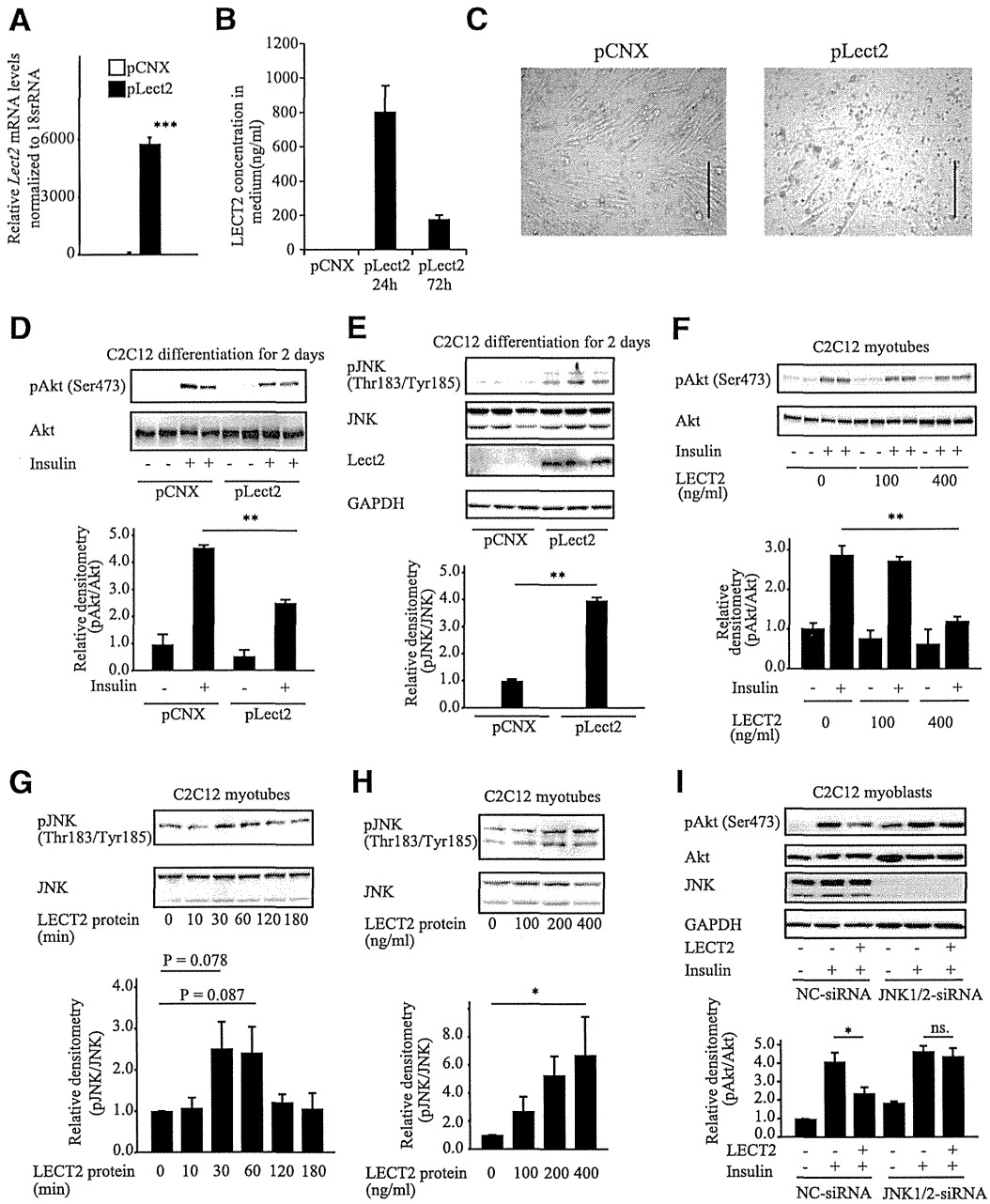

Figure 6—LECT2 impairs insulin signaling by activating JNK in C2C12 myotubes. C2C12 myoblasts in 30–50% confluence were transfected with negative control or mouse (m) Lect2 expression plasmid in *A–E*. When the cells reached 100% confluence, they were differentiated into myotubes with DMEM containing 2% horse serum for 24–48 h. *A*: *Lect2* mRNA levels in C2C12 myotubes transfected with control or *Lect2* expression vector (n = 6). mRNA was obtained from the cells differentiated into myotubes for 24 h. *B*: LECT2 protein levels in culture medium of C2C12 myotubes transfected with control or *Lect2* expression vector for 24 or 72 h (n = 3). LECT2 production was measured by ELISA. *C*: Representative images of C2C12 myotubes transfected with control or *Lect2* expression vector. The cells were differentiated into myotubes for 48 h. *D*: Western blot analysis of phosphorylated Akt in C2C12 myotubes transfected with control or *Lect2* expression vector (n = 4). The cells were stimulated by 100 ng/mL of insulin for 15 min. *E*: Western blot analysis of phosphorylated JNK in C2C12 myotubes transfected with control or *Lect2* expression vector (n = 3). *F*: Western blot analysis of phosphorylated Akt in C2C12 myotubes pretreated with recombinant LECT2 protein (n = 4). The cells were pretreated with LECT2 protein. Three hours later, the cells were stimulated with insulin. *G*: Effects of recombinant LECT2 protein on JNK phosphorylation in C2C12 myotubes (n = 3). The cells were treated with 400 ng/mL of recombinant LECT2 protein for the indicated times. *H*: Concentration-dependent effects of recombinant LECT2 protein on JNK phosphorylation in C2C12 myotubes (n = 3). The cells were treated with LECT2 protein for 1 h. *I*: Effects of JNK-knockdown on LECT2 protein–induced insulin resistance in C2C12 myoblasts (n = 4). Data in *A*, *B*, and *D–I* represent the means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus cells transfected with control vector or cells treated with vehicle.

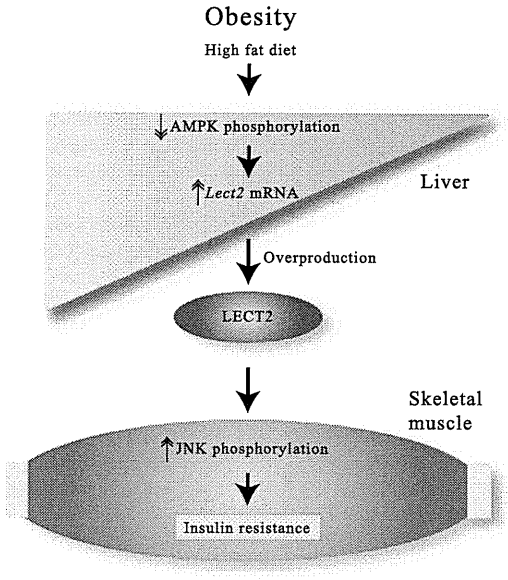

Figure 7—The hepatokine LECT2 links obesity to insulin resistance in skeletal muscle.

the possibility that LECT2-induced suppression of myotube differentiation causes secondary insulin resistance in pLect2 experiments. The results obtained from the experiments using the recombinant LECT2 protein suggest that LECT2 directly induces insulin resistance in C2C12 cells independent of its action on myotube differentiation.

Okumura et al. (55) reported that treatment with LECT2 ameliorated collagen antibody–induced arthritis in mice. This report suggests that LECT2 suppresses the inflammatory response that progresses after autoantibodies develop. Several clinical studies showed that the onset of inflammatory polyarthritis, such as rheumatoid arthritis, is accelerated by obesity (58,59). Because our current data reveal a positive correlation between BMI and serum LECT2 levels in humans, it seems that overproduction of LECT2 fails to exert a sufficient suppressive action on inflammatory polyarthritis in people with obesity. However, it is still unknown whether LECT2 acts on the process of autoantibody production by B lymphocytes in the acquired immune system. Further basic and clinical studies are needed to investigate the relationship between LECT2 and obesity-associated arthritis.

Our current cross-sectional data show that serum levels of LECT2 positively correlate with the severity of insulin resistance in human subjects. However, many lines of evidence demonstrated that various circulating proteins whose expression is altered in obesity, such as adiponectin and resistin, participate in the development of insulin resistance (60). Hence our study does not necessarily place LECT2 as only a single causal factor of insulin resistance. In addition, further prospective studies are needed to confirm the causal relationship between LECT2 and insulin resistance in people with obesity.

In summary, our experiments identified LECT2 as an obesity-upregulated hepatokine that induces insulin resistance in skeletal muscle. *Lect2* may be a potential target for the treatment of obesity-associated insulin resistance.

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Author Contributions. F.L. researched the data and wrote the manuscript. H.M. conceived and designed the experiments, researched the data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. K.C., H.Tak., A.K., K.M., N.T., H.H., N.M.-N., Y.T., H.N., Y.M., T.N., M.N., K.Ta., T.S., K.I., K.To., and Y.S. researched the data. T.O., K.-i.M., S.M., H.Tan., and S.Y. contributed to the discussion. S.K. and T.T. contributed the discussion, wrote the manuscript, and reviewed and edited the manuscript. T.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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ARTICLE

The effects of ezetimibe on non-alcoholic fatty liver disease and glucose metabolism: a randomised controlled trial

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Abstract

Aims/hypothesis The cholesterol absorption inhibitor ezetimibe has been shown to ameliorate non-alcoholic fatty liver disease (NAFLD) pathology in a single-armed clinical study and in experimental animal models. In this study, we investigated the efficacy of ezetimibe on NAFLD pathology in an open-label randomised controlled clinical trial.

Methods We had planned to enrol 80 patients in the trial, as we had estimated that, with this sample size, the study would have 90% power. The study intervention and enrolment were discontinued because of the higher proportion of adverse events (significant elevation in HbA_{1c}) in the ezetimibe group than in the control group. Thirty-two patients with NAFLD were enrolled and randomised (allocation by computer program). Ezetimibe (10 mg/day) was given to 17 patients with NAFLD for 6 months. The primary endpoint was change in serum aminotransferase level. Secondary outcomes were change in liver histology (12 control and 16 ezetimibe patients), insulin sensitivity including a hyperinsulinaemic–euglycaemic clamp study (ten control and 13 ezetimibe patients) and hepatic fatty acid composition (six control and nine ezetimibe patients). Hepatic gene expression profiling was completed in 15 patients using an Affymetrix gene chip. Patients and the physician in charge knew to which group the patient had been allocated, but people carrying out measurements or examinations were blinded to group.

Results Serum total cholesterol was significantly decreased in the ezetimibe group. The fibrosis stage and ballooning score were also significantly improved with ezetimibe treatment. However, ezetimibe treatment significantly increased HbA_{1c} and was associated with a significant increase in hepatic long-chain fatty acids. Hepatic gene expression analysis showed coordinate downregulation of genes involved in skeletal muscle development and cell adhesion molecules in the ezetimibe treatment group, suggesting a suppression of stellate cell development into myofibroblasts. Genes involved in the L-carnitine pathway were coordinately downregulated by ezetimibe treatment and those in the steroid metabolism pathway upregulated, suggestive of impaired oxidation of long-chain fatty acids.

Conclusions/interpretation Ezetimibe improved hepatic fibrosis but increased hepatic long-chain fatty acids and HbA_{1c} in patients with NAFLD. These findings shed light on previously unrecognised actions of ezetimibe that should be examined further in future studies.

Trial registration University Hospital Medical Information Network (UMIN) Clinical Trials Registry UMIN000005250.

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Keywords Ezetimibe · Fatty acid · Gene expression · Non-alcoholic fatty liver disease

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Abbreviations

ALT	Alanine aminotransferase
H-IR	Hepatic insulin resistance index
hsCRP	High-sensitivity C-reactive protein
ICG15	Indocyanine green retention rate at 15 min after venous administration
LXR	Liver-X-receptor
MCR	Glucose metabolic clearance rate
miR	MicroRNA
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NPC1L1	Niemann–Pick C1-like 1
PAI-1	Plasminogen activator inhibitor-1
RLP-C	Remnant-like particle cholesterol
sdLDL	Small dense LDL
SREBP	Sterol regulatory element binding protein
QUICKI	Quantitative insulin sensitivity check index

Introduction

Multiple metabolic disorders, such as diabetes [1], insulin resistance and dyslipidaemia [2], are associated with non-alcoholic fatty liver disease (NAFLD), ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH). Steatosis of the liver is closely associated with insulin resistance. However, the toxic lipids are not intrahepatic triacylglycerols but, rather, it is non-esterified cholesterol [3, 4] and some NEFA [5] that contribute to inflammation and insulin resistance in hepatocytes.

The level of cholesterol is tightly regulated by endogenous synthesis in the liver and dietary absorption/biliary reabsorption in the small intestine. Niemann–Pick C1-like 1 (NPC1L1) plays a pivotal role in cholesterol incorporation in enterocytes [6]. Ezetimibe, a potent inhibitor of cholesterol absorption, inhibits NPC1L1-dependent cholesterol transport at the brush border of the intestine and the liver [6]. This suggests that ezetimibe ameliorates toxic-lipid-induced inflammation and insulin resistance by inhibiting cholesterol absorption. Indeed, ezetimibe improves liver steatosis and insulin resistance in mice [7] and Zucker obese fatty rats [8], although the beneficial effects of ezetimibe are observed only when the animals are fed a high-fat diet. Ezetimibe can also ameliorate liver pathology in patients with NAFLD [9, 10]; however, these studies lack a control group, which precludes meaningful conclusions as liver pathology can improve over the natural course of the disease or with tight glycaemic control in some NAFLD patients [1]. In the present study, we investigated the efficacy of ezetimibe treatment in patients with NAFLD for 6 months in an open-label randomised control study by examining liver pathology, as well as hepatic enzymes, glucose metabolism, hepatic fatty acid composition and hepatic gene expression profiles.

Methods

Patient selection Study staff recruited participants from outpatients at Kanazawa University Hospital, Ishikawa, Japan. Patients were recruited from April 2008 to August 2010, with follow-up visits during the 6 months thereafter. The study lasted from April 2008 to February 2011.

The inclusion criterion was a biopsy consistent with the diagnosis of NAFLD. Exclusion criteria included hepatic virus infections (hepatitis C virus [HCV] RNA–PCR-positive, hepatitis B and C, cytomegalovirus and Epstein–Barr virus), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, haemochromatosis, α_1-antitrypsin deficiency, Wilson's disease, history of parenteral nutrition and use of drugs known to induce steatosis (e.g. valproate, amiodarone and prednisone) or hepatic injury caused by substance abuse and/or the current or past consumption of more than 20 g of alcohol daily. None of the patients had any clinical evidence of hepatic decompensation, such as hepatic encephalopathy, ascites, variceal bleeding or an elevated serum bilirubin level more than twofold the upper normal limit.

A random allocation sequence was computer-generated elsewhere and assigned participants in a 1:1 ratio to treatment with ezetimibe or to the control group. All patients and responsible guardians underwent an hour of nutritional counselling by an experienced dietitian before starting the 6 month treatment period. The experienced dietitians were unaware of the study assignments. In addition, all patients were given a standard energy diet (125.5 kJ/kg per day; carbohydrate 50–60%, fat 20–30%, protein 15–20%) and exercise (5–6 metabolic equivalent estimations for 30 min daily) counselling before the study. Patients remained on stable doses of medications for the duration of the study. The patients in the ezetimibe group received generic ezetimibe (10 mg/day; Zetia, [Merck, Whitehouse Station, NJ, USA]) for 6 months.

The study was conducted with the approval of the Ethics Committee of Kanazawa University Hospital, Ishikawa, Japan, in accordance with the Declaration of Helsinki. Written informed consent was obtained from all individuals before enrolment. This trial is registered with the University Hospital Medical Information Network (UMIN) (Clinical Trials Registry, no. UMIN000005250).

Primary and secondary outcomes The primary endpoint was change in serum alanine aminotransferase (ALT) level at month 6 from baseline. Secondary outcomes included changes in the histological findings for NAFLD, hepatic gene expression profiling, fatty acid compositions of plasma and liver biopsy samples, lipid profiles, insulin resistance and

anthropometric measures, as well as assessment of ezetimibe safety. We had planned to enrol 80 patients in the trial, as we had estimated that with this sample size, the study would have 90% power at an α (two-tailed) value of 0.05 showing a 50% decrease of serum ALT values with 6 months of pioglitazone therapy on the basis of a previous study [11]. At the time of adverse event analyses, 32 of the targeted 80 patients had been randomly assigned and were included in the safety analyses.

Data collection Clinical information, including age, sex and body measurements, was obtained for each patient. Venous blood samples were obtained after the patients had fasted overnight (12 h) and were used to evaluate blood chemistry. Insulin resistance was estimated by HOMA-IR, calculated as [fasting insulin (pmol/l) × fasting glucose (mmol/l)]/22.5 [12] and insulin sensitivity was estimated as the quantitative insulin sensitivity check index (QUICKI)[13]. The adipose tissue insulin resistance index (adipose IR) was calculated as fasting NEFA (mmol/l)×fasting insulin (pmol/l) [14–16]. The indocyanine green retention rate at 15 min after venous administration (ICG15) was assessed using standard laboratory techniques before and after treatment. Serum fatty acids were measured with a gas chromatograph (Shimizu GC 17A, Kypto, Japan) at SRL (Tokyo, Japan).

Evaluation of insulin sensitivity derived from an OGTT After an overnight fast (10–12 h), a 75 g OGTT was performed at 08:30 hours. The OGTT-derived index of beta cell function, the insulinogenic index, computed as the suprabasal serum insulin increment divided by the corresponding plasma glucose increment in the first 30 min (ΔI30/ΔG30) [15, 17, 18] was calculated. From the OGTT data, the Matsuda index [19] was calculated. The hepatic insulin resistance index (H-IR) was calculated as the product of the total AUCs for glucose and insulin during the first 30 min of the OGTT (glucose 0–30 [AUC] [mmol/l]×insulin 0–30 [AUC] [pmol/l]). Skeletal muscle insulin sensitivity can be calculated as the rate of decline in plasma glucose concentration divided by plasma insulin concentration, as follows. Muscle insulin sensitivity index=dG/dt/mean plasma insulin concentration, where dG/dt is the rate of decline in plasma glucose concentration and is calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir [20]. See the electronic supplementary material (ESM) for further details.

Evaluation of insulin sensitivity derived from the euglycaemic insulin clamp Insulin sensitivity in 23 of the 31 patients (10 control and 13 ezetimibe patients) was also evaluated in a hyperinsulinaemic–euglycaemic clamp study [21]. Patients did not receive any medication on the morning of the examination. At ~09:00 hours, after an overnight fast of at least 10 h, an intravenous catheter was placed in an antecubital vein in each individual for infusion, while a second catheter was placed in the contralateral hand for blood sampling. The euglycaemic–hyperinsulinaemic clamp technique was performed using an artificial pancreas (model STG-22; Nikkiso, Tokyo, Japan), as described previously [22]. See ESM for further details. The mean glucose metabolic clearance rate (MCR) in healthy individuals (n=9; age, 26.60± 2.9 years; body mass index, 22.3±2.1 kg/m^2) was 13.5± 3.4 mg kg^{-1} min^{-1} [2].

Liver biopsy pathology A single pathologist, who was blinded to the clinical information and the order in which the biopsies were obtained, analysed all biopsies twice and at separate times. The sections were cut from a paraffin block and stained with haematoxylin and eosin, Azan–Mallory and silver reticulin impregnation. The biopsied tissues were scored for steatosis (from 0 to 3), stage (from 1 to 4) and grade (from 1 to 3) as described [2], according to the standard criteria for grading and staging of NASH proposed by Brunt et al [23]. The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3) and ballooning (0–2), as reported by Kleiner et al [24].

Gene expression analysis of liver biopsied samples Gene expression profiling was performed in samples from nine patients in the ezetimibe group and six in the control group. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. See ESM for further details. Data files (CEL) were obtained using the GeneChip Operating Software 1.4 (Affymetrix). Genechip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). The data were log-transformed ($\log_{10}$), normalised and centred. To identify genetic variants, paired t tests were performed to define p values <0.05 and fold change>1.5. Pathway analysis was performed using MetaCore (GeneGo, St Joseph, MI, USA). Functional ontology enrichment analysis was performed to compare the gene ontology (GO) process distribution of differentially expressed genes ($p < 0.01$).

Fatty acid composition of liver Aliquots (0.2 mg) of liver samples snap-frozen by liquid nitrogen were homogenised in 1 ml normal NaCl solution (NaCl 154 mmol/l). Briefly, fatty acids were extracted by using pentadecanoic acid, and saponified with alkaline reagent (0.5 mmol/l KOH/ CH_3OH). The fatty acid methyl esters were analysed in a gas chromatograph (Shimadzu GC-2014 AF/SPL; Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector and an auto injector. See ESM for further details. Mass spectra were analysed using GC solution (v. 2.3) software (Shimadzu Corporation, Kyoto, Japan, www.shimadzu.com). The changes in hepatic fatty acid composition are expressed as 10^{-4} mg/mg liver.

Statistical analysis Data are expressed as mean ± one standard error, unless indicated otherwise. The Statistical Package for the Social Sciences (SPSS; version 11.0; Chicago, IL, USA) was used for the statistical analyses. For univariate comparisons between the patient groups, Student's *t* test or Mann–Whitney's *U* test was used, as appropriate, followed by the Bonferroni multiple-comparison test. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Enrolment and discontinuation The data and safety monitoring board recommended that the study intervention and enrolment be discontinued because of the higher proportion of adverse events (significant elevation in HbA_{1c}) in the ezetimibe group than in the control group. At the time of adverse event analyses, 32 of the targeted 80 patients had been randomly assigned and were included in the safety analyses. In our open-label trial, 32 patients with NAFLD were enrolled. They were randomised to treatment with ezetimibe ($n=17$) or a control ($n=15$) with no significant clinical differences in variables between the groups. Of the 32 randomly assigned patients, 31 had completed the 6 month intervention period; one patient dropped out of the study. One case in the control group withdrew consent after randomisation and before intervention (ESM Fig. 1). The patient who withdrew was excluded from analysis because he did not start his course of treatment. Two analyses were conducted in the remaining patients. In the intention-to-treat analysis (ESM Tables 1 and 2), measures that were missing for participants who discontinued the study were replaced with baseline measures. In the second analysis, the only data included were from participants who completed the study to the end of the 6 month follow-up period. We performed a completed case analysis because there were few dropouts unrelated to baseline values or to their response.

Patient characteristics The 31 study patients (mean age 52.7±2.1 years; mean BMI 29.2±1.0) included 14 randomised to the control group and 17 to the ezetimibe group (ESM Table 3).

At baseline, the characteristics of patients in the ezetimibe and control groups were comparable except for the waist circumference ($p=0.085$) and the Matsuda index ($p=0.060$). The histological features of the liver are summarised in Table 1. At baseline, neither the severity of the individual histological features nor the proportion of patients distributed in the three NAS categories was significantly different between the two groups. All 31 participants agreed to complete the follow-up venous blood samples including OGTT. The ICG15 was conducted in 24 patients (ten control and 14 ezetimibe patients).

Changes in laboratory variables The primary study outcome, serum alanine aminotransferase levels, did not change after ezetimibe treatment (Table 2).

After 6 months of ezetimibe treatment, systolic blood pressure, HbA_{1c}, glycated albumin, and lathosterol were significantly increased, while total cholesterol levels, campesterol, sitosterol and ferritin were significantly decreased. In contrast, body weight, BMI, fasting plasma glucose, plasma γ-glutamyltransferase, triacylglycerols, HDL-cholesterol, small dense LDL (sdLDL), remnant-like particle cholesterol (RLP-C), type IV collagen 7 s levels, NEFA, total bile acid, high-sensitivity C-reactive protein (hsCRP), adiponectin, TNF-α, plasminogen activator inhibitor-1 (PAI-1), 8-isoprostanes and ICG15 did not change after ezetimibe treatment (Table 2). Adipose IR tended to increase in the ezetimibe group (from 88.1±25.5 to 107.5±25.5, $p=0.070$), but not in the control group.

When changes in the groups were compared, the ezetimibe group, but not the control group, had a significant decrease in total cholesterol (ezetimibe, −0.49±0.19 vs control, 0.06±0.14 mmol/l; $p=0.037$), whereas the ezetimibe group, but not control group, showed a significant elevation in HbA_{1c} (ezetimibe, 0.46±0.12% [4.95±1.28 mmol/mol] vs control, 0.08±0.13% [0.78±1.46 mmol/mol]; $p=0.041$). Also, there were significant differences between the groups in cholesterol and HbA_{1c} levels at 6 months. The multiple-comparison

Table 1 Histological characteristics of the livers of patients who completed the study at baseline and 6 months

Variable	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
Steatosis	1.42±0.15	1.17±0.17	0.082	1.56±0.18	1.31±0.15	0.300	0.989
Stage	1.71±0.40	1.71±0.39	1.000	1.75±0.28	1.53±0.26	0.048	0.163
Grade	0.88±0.28	0.79±0.26	0.339	0.84±0.21	0.72±0.15	0.362	0.628
Acinar inflammation	0.88±0.20	0.83±0.20	0.674	1.00±0.13	0.97±0.13	0.751	0.060
Portal inflammation	0.67±0.19	0.71±0.13	0.795	0.44±0.16	0.56±0.16	0.333	0.941
Ballooning	0.58±0.23	0.58±0.23	1.000	0.69±0.20	0.41±0.15	0.045	0.677
NAFLD activity score	3.25±0.53	2.82±0.59	0.139	3.71±0.50	3.06±0.45	0.185	0.705

Data are expressed as the means ± SE

[a] p value for the intergroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

Table 2 Laboratory values, insulin sensitivity and insulin resistance derived from the euglycaemic insulin clamps and OGTTs of patients who completed the study at baseline and 6 months

Variable	Control			Ezetimibe			
	Before	After	p^a	Before	After	p^a	p^b
Male/female	9/5			11/6			0.232
Age (years)	55.5±3.0			50.4±2.9			
Body weight (kg)	74.4±6.2	73.0±5.6	0.144	81.5±4.6	80.1±4.2	0.367	0.983
BMI (kg/m^2)	27.7±1.7	27.3±1.5	0.172	30.5±1.2	30.0±1.1	0.383	0.999
Waist circumference (cm)	93.1±2.7	92.6±3.4	0.709	99.9±2.5	100.0±2.6	0.956	0.713
Systolic blood pressure (mmHg)	125.2±3.9	126.4±4.9	0.771	124.0±2.4	130.7±2.8	0.048	0.269
Fasting plasma glucose (mmol/l)	7.15±0.63	6.52±0.40	0.240	6.62±0.30	6.87±0.34	0.411	0.131
HbA_{1c} (%)	5.9±0.2	6.0±0.2	0.603	6.1±0.2	6.5±0.2	0.001	0.041
HbA_{1c} (mmol/mol)	40.8±2.2	41.6±2.6	0.603	43.0±2.6	48.0±2.3	0.001	0.041
Hepaplastin test (%)	115.9±5.8	117.1±6.4	0.624	113.7±4.6	111.8±3.7	0.583	0.459
Glycated albumin (%)	15.9±0.8	16.2±1.0	0.397	15.7±0.5	16.8±0.5	0.014	0.196
Serum aspartate aminotransferase (μkat/l)	31.1±4.4	30.3±3.0	0.780	41.8±6.7	33.7±4.1	0.252	0.365
Serum ALT (μkat/l)	37.9±6.8	38.0±4.5	0.978	53.2±8.6	49.3±6.5	0.683	0.723
Plasma γ-glutamyltransferase (μkat/l)	74.9±27.8	65.8±19.5	0.345	71.4±23.4	60.5±16.1	0.220	0.892
Total cholesterol (mmol/l)	5.14±0.21	5.20±0.18	0.672	5.14±0.20	4.65±0.17	0.024	0.037
Triacylglycerols (mmol/l)	1.34±0.12	1.17±0.12	0.105	1.43±0.11	1.46±0.13	0.857	0.303
HDL-C (mmol/l)	1.40±0.08	1.45±0.06	0.914	1.36±0.08	1.36±0.06	0.942	0.903
sdLDL (mmol/l)	0.52±0.07	0.54±0.07	0.782	0.61±0.10	0.50±0.06	0.201	0.251
RLP-C (mmol/l)	0.13±0.01	0.11±0.01	0.163	0.12±0.01	0.11±0.01	0.601	0.365
Lathosterol×10^{-3} (μmol/l)	2.27±0.43	2.85±0.52	0.001	3.52±0.52	5.01±0.67	<0.001	0.018
Campesterol×10^{-3} (μmol/l)	4.32±0.65	6.20±0.68	0.004	3.78±0.42	2.49±0.30	0.007	<0.001
Sitosterol×10^{-3} (μmol/l)	3.04±0.47	3.89±0.39	0.079	2.73±0.28	1.81±0.19	0.004	0.002
Ferritin (pmol/l)	412.1±85.6	235.3±47.0	0.009	395.7±81.3	247.8±56.8	0.005	0.689
Type IV collagen 7 s (μg/l)	4.52±0.48	4.42±0.45	0.622	4.23±0.23	4.33±0.20	0.592	0.465
NEFA (mmol/l)	0.50±0.09	0.63±0.06	0.160	0.51±0.05	0.57±0.03	0.835	0.447
Total bile acid (μmol/l)	12.5±8.0	8.8±5.2	0.214	5.0±0.7	4.8±1.3	0.893	0.267
hsCRP×10^{-3} (μg/ml)	0.12±0.02	0.09±0.02	0.050	0.14±0.04	0.13±0.04	0.886	0.767
Adiponectin (μg/ml)	4.0±0.5	4.6±0.8	0.114	3.0±0.6	3.3±0.6	0.299	0.670
TNF-α ×10^{-5} (pmol/ml)	10.4±2.3	15.6±8.1	0.094	8.1±0.6	30.0±12.7	0.183	0.084
Leptin×10^{-3} (μg/l)	8.1±1.0	9.7±1.3	0.044	10.8±1.4	12.4±1.5	0.085	0.982
PAI-1 (pmol/l)	400.0±44.2	436.5±44.2	0.401	550.0±71.2	488.5±67.3	0.217	0.136
8-Isoprostanes (pmol/mmol creatinine)	76.9±14.3	57.0±8.0	0.147	56.5±6.6	68.0±7.7	0.092	0.031
ICG15 (%)	8.7±2.4	8.5±2.0	0.662	7.7±1.7	7.7±1.5	0.984	0.796
HOMA-IR	10.1±6.5	5.0±2.1	0.471	9.5±2.6	9.3±2.2	0.839	0.479
QUICKI	0.32±0.01	0.33±0.01	0.443	0.30±0.01	0.30±0.01	0.984	0.019
Adipose IR	55.8±15.5	78.8±31.7	0.441	88.1±25.5	107.5±25.5	0.070	0.099
Insulinogenic index	0.43±0.09	0.53±0.11	0.307	0.41±0.08	0.35±0.09	0.501	0.765
H-IR×10^6	1.82±0.46	2.29±0.44	0.568	2.29±0.33	2.66±0.41	0.221	0.796
Matsuda index	3.03±0.45	3.35±0.49	0.368	1.99±0.28	2.01±0.29	0.895	0.013
Muscle insulin sensitivity	0.039±0.006	0.058±0.016	0.210	0.036±0.005	0.034±0.004	0.560	0.067
MCR	4.86±0.50	4.36±0.45	0.174	4.70±0.31	4.80±0.35	0.827	0.352

Data are expressed as means ± SE

[a] p value for the intergroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

HDL-C, HDL-cholesterol

Table 3 Signalling pathway gene expression changes in the ezetimibe group

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
Development_skeletal muscle development	*VEGFA*	Vascular endothelial growth factor A	210512_s_at	Down	Angiogenesis
	ACTA2	Actin, α2, smooth muscle, aorta1	200974_at	Down	Cytoskeleton and cell attachment
	TCF3	Transcription factor 3	209153_s_at	Down	Differentiation
	TTN	Titin	1557994_at	Down	Abundant protein of striated muscle
	TPM2	Tropomyosin 2	204083_s_at	Down	Actin filament binding protein
	MYH11	Myosin, heavy chain 11, smooth muscle	201496_x_at	Down	Smooth muscle myosin
Immune response_phagocytosis	*FYB*	FYN-binding protein	205285_s_at	Up	Platelet activation and IL2 expression
	FCGR3A	Fc fragment of IgG, low affinity IIIA	204006_s_at	Up	ADCC and phagocytosis
	LCP2	Lymphocyte cytosolic protein 2	244251_at	Up	T cell antigen receptor mediated signalling
	CLEC7A	C-type lectin domain family 7, member A	221698_s_at	Up	T cell proliferation
	MSR1	Macrophage scavenger receptor 1	214770_at	Up	Macrophage-associated processes
	FCGR2A	Fc fragment of IgG, low affinity IIA	1565673_at	Up	Promotes phagocytosis
	PRKCB	Protein kinase C, β	209685_s_at	Up	B cell activation, apoptosis induction
	PLCB4	Phospholipase C, β4	240728_at	Up	Inflammation, cell growth, signalling and death
Cell adhesion_integrin priming	*GNA12*	G protein α12	221737_at	Down	Cytoskeletal rearrangement
	ITGB3	Integrin, β3	204628_s_at	Down	Ubiquitously expressed adhesion molecules
	PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2	229392_s_at	Down	Diverse range of cell functions
Cell adhesion_cadherins	*PTPRF*	Protein tyrosine phosphatase, receptor type, F	200636_s_at	Down	Cell adhesion receptor
	BTRC	β-Transducin repeat containing E3 ubiquitin protein ligase	222374_at	Down	Substrate recognition component of a SCF E3 ubiquitin-protein ligase complex
	CDHR2	Cadherin-related family member 2	220186_s_at	Down	Contact inhibition at the lateral surface of epithelial cells
	SKI	V-ski sarcoma viral oncogene homologue	229265_at	Down	Repressor of TGF-β signalling
	MLLT4	Myeloid/lymphoid or mixed-lineage leukaemia	214939_x_at	Down	Belongs to an adhesion system
	VLDLR	Very low density lipoprotein receptor	209822_s_at	Down	Binds VLDL and transports it into cells by endocytosis
O-Hexadecanoyl-L-carnitine pathway	*TUBB2B*	Tubulin, β 2B class IIB	209372_x_at	Down	Major component of microtubules
	TUBB2A	Tubulin, β2A class IIA	209372_x_at	Down	Major component of microtubules
	PLCE1	Phospholipase C, epsilon 1	205112_at	Down	Hydrolyses phospholipids into fatty acids and other lipophilic molecules
	CPT1B	Carnitine palmitoyltransferase 1B (muscle)	210070_s_at	Down	Rate-controlling enzyme of the long-chain fatty acid β-oxidation pathway
	CPT1A	Carnitine palmitoyltransferase 1A (liver)	203634_s_at	Down	Carnitine-dependent transport across the mitochondrial inner membrane
	NR1H4	Nuclear receptor subfamily 1, group H, member 4	243800_at	Down	Involved in bile acid synthesis and transport.
GalNAcbeta1-3Gal pathway	*PLCB4*	Phospholipase C, β4	240728_at	Up	Formation of inositol 1,4,5-trisphosphate and diacylglycerol
Steroid metabolism_cholesterol biosynthesis	*CYP51A1*	Cytochrome P450, family 51, subfamily A, polypeptide 1	216607_s_at	Up	Transforms lanosterol
	SREBF2	Sterol regulatory element binding transcription factor 2	242748_at	Up	Transcriptional activator required for lipid homeostasis
	SQLE	Squalene epoxidase	209218_at	Up	Catalyses the first oxygenation step in sterol biosynthesis

Table 3 (continued)

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
	SC5DL	Sterol-C5-desaturase-like	215064_at	Up	Catalyses the conversion of lathosterol into 7-dehydrocholesterol
	HMGCS1	3-Hydroxy-3-methylglutaryl-CoA synthase 1	205822_s_at	Up	Condenses acetyl-CoA with acetoacetyl-CoA to form HMG-CoA

Bonferroni test revealed highly significant differences in the changes in total cholesterol ($p = 0.037$) and HbA_{1c} ($p = 0.040$) between the ezetimibe and control groups.

Increased concentrations of the cholesterol synthesis markers lathosterol (ezetimibe, 1.49±0.32 nmol/l vs control, 0.58±0.14 nmol/l; $p = 0.018$) and decreased concentrations of the cholesterol absorption markers campesterol (ezetimibe, −1.28±0.41 nmol/l vs control, 1.88±0.54 nmol/l, $p = 0.000$) and sitosterol (ezetimibe, −0.91±0.27 nmol/l vs control, 0.85± 0.45 nmol/l; $p = 0.002$) were observed on treatment. The ezetimibe group had an increase, whereas the control group had a decrease, in the level of 8-isoprostanes (ezetimibe, 11.6± 6.4 pmol/mmol creatinine vs control, −19.9±12.9 pmol/mmol creatinine; $p = 0.031$).

When changes between groups were compared, the ezetimibe group had a greater decrease in the Matsuda index (ezetimibe=−0.78±0.57 vs control=−1.35±0.55, $p = 0.013$), QUICKI (ezetimibe= −0.02±0.01 vs control=0.03±0.0, $p = 0.019$), and muscle insulin sensitivity (ezetimibe= −0.002±0.004 vs control=0.019±0.014, $p = 0.067$) than the control group.

Changes in liver histology Twenty-eight of 31 participants, 16 in the ezetimibe group and 12 in the control group, agreed to complete the follow-up and undergo a liver biopsy at 6 months, allowing for complete case analysis of the data (Table 1). After 6 months, the changes in staging score (from 1.75±0.28 to 1.53±0.26) and ballooning score (from 0.69± 0.20 to 0.41±0.15) were significantly improved in the ezetimibe group compared with the control group, whereas the scores of steatosis, lobular inflammation and NAS were not significantly changed in either group. The degree of all of these histological features was not significantly different between the two groups (Table 1).

Serial changes in liver gene with ezetimibe treatment Gene expression profiling was conducted in samples from nine patients in the ezetimibe group and six in the control group (ESM Table 4). In the ezetimibe group, 434 genes were upregulated and 410 genes downregulated, while in the control group, 643 genes were upregulated and 367 genes downregulated. Pathway analysis of the process network of differentially expressed genes showed coordinate downregulation of genes involved in skeletal muscle development and cell adhesion molecules in the ezetimibe group, suggesting a suppression of stellate cell development into myofibroblasts (Table 3). In addition, ezetimibe activated the immune response pathway. In contrast, genes involved in skeletal muscle development were upregulated and those in the immune response downregulated in the control group (Table 4). Pathway analysis of the metabolic network also revealed decreased L-carnitine pathway and increased steroid metabolism with ezetimibe treatment, but decreased CoA biosynthesis and increased glycerol 3-phosphate pathway in the control group (ESM Fig. 2).

Changes in plasma fatty acid composition and fatty acid composition extracted from liver tissue The changes in plasma fatty acid composition are shown in Table 5. Compared with baseline levels, only eicosatrienoic acid was significantly increased in the ezetimibe group.

Fatty acid composition in extracted liver tissue was available for 16 NAFLD patients treated with ezetimibe and 12 controls (Table 6). Ezetimibe treatment for 6 months significantly and markedly increased hepatic lauric, myristic, palmitic, palmitoleic, margaric and stearic acids compared with the control group. The changes in hepatic fatty acid composition did not correlate with the changes in serum fatty acid composition before and after ezetimibe treatment (ESM Table 5).

Discussion

This is the first report of the efficacy of ezetimibe treatment on liver pathology in patients with NAFLD in an open-label randomised controlled trial. Treatment with 10 mg/day ezetimibe for 6 months did not alter the primary study outcome, serum aminotransferase levels. Ezetimibe significantly decreased serum cholesterol levels and cholesterol absorption markers as expected, whereas, in contrast to previous reports, ezetimibe treatment did not decrease serum levels of triacylglycerol. Our initial hypothesis was that ezetimibe treatment ameliorates liver pathology by inhibiting the absorption of toxic lipids such as oxidised cholesterol and palmitate. In our animal model, cholesterol feeding to mice increased not

Table 4 Signalling pathway gene expression changes in the control group

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
Muscle contraction	*MYH11*	Myosin, heavy chain 11, smooth muscle	201497_x_at	Up	Smooth muscle myosin
	CALM1	Calmodulin 1	241619_at	Up	Ion channels and other proteins by Ca^{2+}
	KCNJ15	Potassium inwardly-rectifying channel, subfamily J, member 15	211806_s_at	Up	Integral membrane protein, inward-rectifier type potassium channel
	SRI	Sorcin	208920_at	Up	Modulates excitation–contraction coupling in the heart
	ACTA2	Actin, α2, smooth muscle, aorta	215787_at	Up	Cell motility, structure and integrity
	TTN	Titin	1557994_at	Up	Abundant protein of striated muscle
	EDNRA	Endothelin receptor type A	204463_s_at	Up	Receptor for endothelin-1
	TPM2	Tropomyosin 2	204083_s_at	Up	Actin filament binding protein
	CRYAB	Crystallin, αB	209283_at	Up	Transparency and refractive index of the lens
Development_skeletal muscle development	*GTF2IRD1*	GTF2I repeat domain containing 1	218412_s_at	Up	Transcription regulator involved in cell-cycle progression, skeletal muscle differentiation
	ADAM12	ADAM metallopeptidase domain 12	213790_at	Up	Skeletal muscle regeneration
	MAP1B	Microtubule-associated protein 1B	226084_at	Up	Facilitates tyrosination of α-tubulin in neuronal microtubules
	MYOM1	Myomesin 1	205610_at	Up	Major component of the vertebrate myofibrillar M band
Cell cycle_G1-S growth factor regulation	*DACH1*	Dachshund homologue 1	205472_s_at	Up	Transcription factor that is involved in regulation of organogenesis
	FOXN3	Forkhead box N3	229652_s_at	Up	Transcriptional repressor, DNA damage-inducible cell cycle arrests
	TGFB2	Transforming growth factor, β2	228121_at	Up	Suppressive effects on interleukin-2 dependent T cell growth
	PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta	203879_at	Up	Generate PIP3, recruiting PH domain-containing proteins to the membrane
	EGFR	Epidermal growth factor receptor	1565484_x_at	Up	Antagonist of EGF action
	CCNA2	Cyclin A2	203418_at	Up	Control of the cell cycle at the G1/S and the G2/M transitions
	AKT3	v-Akt murine thymoma viral oncogene homologue 3	219393_s_at	Up	Metabolism, proliferation, cell survival, growth and angiogenesis
	PRKD1	Protein kinase D1	205880_at	Up	Converts transient DAG signals into prolonged physiological effects
Regulation of metabolism_	*INSR*	Insulin receptor	226450_at	Down	Pleiotropic actions of insulin
Bile acid regulation of lipid metabolism and	*SLC27A5*	Solute carrier family 27, member 5	219733_s_at	Down	Bile acid metabolism
Negative FXR-dependent regulation of bile acids concentration	*MBTPS2*	Membrane-bound transcription factor peptidase	1554604_at	Down	Intramembrane proteolysis of SREBPs
	PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3	202743_at	Down	During insulin stimulation, it also binds to IRS-1
	MTTP	Microsomal triacylglycerol transfer protein	205675_at	Down	Catalyses the transport of triglyceride, cholesteryl ester, and phospholipid
	PPARA	Peroxisome proliferator-activated receptor α	226978_at	Down	Ligand-activated transcription factor
	CYP7A1	Cytochrome P450, family 7, subfamily A	207406_at	Down	Catalyses cholesterol catabolism and bile acid biosynthesis
	FOXA3	Forkhead box A3	228463_at	Down	Transcription factor

Table 4 (continued)

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
Immune response_phagosome in antigen presentation	*HLA-B*	Major histocompatibility complex, class I, B	211911_x_at	Down	Foreign antigens to the immune system
	CD14	CD14 molecule	201743_at	Down	Mediates the innate immune response to bacterial lipopolysaccharide
	LBP	Lipopolysaccharide binding protein	211652_s_at	Down	Binds to the lipid A moiety of bacterial lipopolysaccharides
	CTSS	Cathepsin S	202901_x_at	Down	Thiol protease
	DERL1	Derlin 1	222543_at	Down	Functional component of endoplasmic reticulum-associated degradation
	CFL2	Cofilin 2	224352_s_at	Down	Reversibly controls actin polymerisation and depolymerisation
	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	230100_x_at	Down	Activated kinase acts on a variety of targets
Vitamin, mediator and cofactor	*SLC1A2*	Solute carrier family 1, member 2	1558009_at	Down	Transports L-glutamate and also L- and D-aspartate
Metabolism_CoA biosynthesis and transport	*PANK3*	Pantothenate kinase 3	218433_at	Down	Physiological regulation of the intracellular CoA concentration
	PANK1	Pantothenate kinase 1	226649_at	Down	Physiological regulation of the intracellular CoA concentration
	VNN1	Vanin 1	205844_at	Down	Membrane-associated proteins
	ACSL5	Acyl-CoA synthetase long-chain family member 5	222592_s_at	Down	Synthesis of cellular lipids and degradation via β-oxidation
	ACOT1	Acyl-CoA thioesterase 1	202982_s_at	Down	Catalyses the hydrolysis of acyl-CoAs to the NEFA and coenzyme A
	ACOT2	Acyl-CoA thioesterase 2	202982_s_at	Down	Catalyses the hydrolysis of acyl-CoAs to the NEFA and coenzyme A
	ENPP1	Ectonucleotide pyrophosphatase/ phosphodiesterase 1	229088_at	Down	Involved primarily in ATP hydrolysis at the plasma membrane
Phatidic acid pathway	*GPR63*	G protein-coupled receptor 63	220993_s_at	Up	Orphan receptor. May play a role in brain function
2-Oleoyl-glycerol_3-phosphate pathway	*LPAR1*	Lysophosphatidic acid receptor 1	204037_at	Up	Receptor for LPA, a mediator of diverse cellular activities

only cholesterol but also triacylglycerols in the liver, and upregulated the gene for sterol regulatory element binding protein (SREBP)-1c that governs fatty acid synthesis [3], probably via activation of liver-X-receptor (LXR) in the liver [25]. Therefore, in experimental models of high-cholesterol-diet-induced steatohepatitis, ezetimibe ameliorated liver steatosis by reducing cholesterol-induced activation of LXR and SREBP-1c [26, 27]. In the present study, however, treatment with ezetimibe unexpectedly ameliorated liver fibrosis staging and ballooning scores without significantly changing hepatic steatosis and insulin resistance.

One possible explanation for the improvement of hepatic fibrosis by ezetimibe treatment may be related to the direct effect of cholesterol on hepatic fibrogenesis. The cholesterol molecule affects membrane organisation and structure, which are critical determinants of membrane bilayer permeability and fluidity [28]. Altered cholesterol metabolism has several toxic effects on hepatocytes, resident macrophages, Kupffer cells and hepatic stellate cells, which promote NASH through diverse mechanisms. Hepatic stellate cells, in particular, are responsible for liver fibrosis in NASH. It has recently been reported that intracellular cholesterol accumulation directly activates hepatic stellated cells through a toll-like receptor-4-dependent pathway and triggers hepatic fibrosis [29]. These effects might be more evident in humans because, unlike rodents, where NPC1L1 is primarily expressed in the intestine, in humans *NPC1L1* mRNA is highly expressed both in the small intestine and liver. Therefore, ezetimibe is estimated to inhibit not only dietary and biliary cholesterol absorption through the small intestine, but also reabsorption of biliary cholesterol in the liver [30, 31]. Thus, ezetimibe may inhibit liver fibrosis by ameliorating

Table 5 Changes in plasma fatty acid composition

Fatty acid	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
C12:0 (lauric acid)	1.9±0.5	1.2±0.2	0.177	2.3±0.6	2.1±0.5	0.753	0.301
C14:0 (myristic acid)	24.9±2.5	23.6±2.9	0.575	27.1±2.8	29.5±3.7	0.441	0.352
C16:0 (palmitic acid)	698.0±24.7	690.0±38.2	0.827	714.3±32.5	717.0±36.2	0.991	0.893
C16:1n-7 (palmitoleic acid)	68.6±6.5	72.5±9.6	0.643	62.4±5.0	69.9±6.2	0.219	0.721
C17:0 (margaric acid)	NE	NE		NE	NE		
C18:0 (stearic acid)	203.3±9.4	196.7±6.9	0.488	207.2±7.7	211.0±9.9	0.854	0.571
C18:1n-9 (oleic acid)	560.2±31.3	556.4±30.3	0.914	547.3±23.9	578.8±32.1	0.475	0.550
C18:2n-6 (linoleic acid)	745.8±26.3	750.6±34.4	0.910	735.8±34.2	713.5±31.4	0.558	0.629
C18:3n-6 (γ-linolenic acid)	9.8±1.3	9.2±1.0	0.506	9.8±0.9	11.1±1.5	0.402	0.300
C18:3n-3 (α-linolenic acid)	21.7±1.6	20.1±1.4	0.285	23.0±2.2	21.6±1.5	0.507	0.924
C20:0n-6 (arachidic acid)	7.0±0.4	6.9±0.3	0.671	7.2±0.2	7.0±0.3	0.410	0.642
C20:1n-9 (eicosenoic acid)	4.8±0.3	4.8±0.4	0.323	4.3±0.2	4.2±0.3	0.831	0.343
C20:2n-6 (eicosadienoic acid)	6.1±0.4	6.1±0.3	0.899	5.6±0.2	5.7±0.3	0.774	0.770
C20:3n-6 (dihomo-γ-linolenic acid)	36.6±3.0	37.3±2.8	0.784	36.5±2.4	40.6±3.7	0.247	0.438
C20:3n-9 (eicosatrienoic acid)	2.5±0.4	2.4±0.4	0.941	1.9±0.2	2.7±0.5	0.034	0.079
C20:4n-6 (arachidonic acid)	135.7±8.4	138.8±6.0	0.689	143.8±11.1	151.1±11.0	0.538	0.787
C20:5n-3 (eicosapentaenoic acid)	67.0±9.0	71.3±9.3	0.640	64.4±7.2	59.1±5.7	0.385	0.369
C22:0 (behenic acid)	16.6±0.8	18.3±1.0	0.035	17.1±0.8	17.9±1.3	0.623	0.468
C22:1n-9 (erucic acid)	1.6±0.1	1.3±0.1	0.066	1.3±0.1	1.3±0.1	0.914	0.170
C22:2n-6 (docosadienoic acid)	NE	NE		NE	NE		
C22:4n-6 (docosatetraenoic acid)	3.9±0.2	4.2±0.2	0.252	4.4±0.3	4.9±0.6	0.262	0.689
C22:5n-3 (docosapentaenoic acid)	20.0±1.4	20.7±1.7	0.657	20.7±1.7	21.5±1.7	0.839	0.887
C22:6n-3 (docosahexaenoic acid)	128.7±9.8	138.6±9.3	0.231	126.5±10.0	128.3±10.8	0.936	0.456
C24:1 (nervonic acid)	35.4±2.2	36.1±2.1	0.656	31.6±1.8	30.3±1.9	0.275	0.263

Data are expressed as means ± SE

[a] p value for the intergroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

NE, not estimated

cholesterol-induced activation of hepatic stellate cells in patients with NAFLD. This hypothesis was well supported by the hepatic gene expression profile induced by ezetimibe administration. Ezetimibe treatment coordinately downregulated genes involved in skeletal muscle development and cell adhesion molecules, suggesting that ezetimibe suppressed stellate cell development into myofibroblasts and thereby inhibited fibrogenesis.

Another important finding of the present study was that treatment with ezetimibe significantly deteriorated glycaemic control. Ezetimibe therapy also altered the hepatic profile of fatty acid components by significantly increasing hepatic levels of lauric, myristic, palmitic, palmitoleic, margaric, stearic, oleic and linoleic acids. Experimentally, palmitate induces interleukin-8 [32], endoplasmic reticulum stress, and c-Jun amino-terminal kinase activation and promotes apoptosis in the liver [5, 33, 34]. Lipid-induced oxidative stress and inflammation are closely related to insulin resistance [3, 5], which could be relevant to the ezetimibe-induced deterioration of glucose homeostasis. Indeed, urinary excretion of 8-isoprostanes was significantly increased in the ezetimibe group compared with the control, and showed significant negative correlation with insulin sensitivity indices such as the Matsuda index and QUICKI in the present study (ESM Table 6). Moreover, hepatic gene expression in the ezetimibe group showed coordinated upregulation of genes involved in the immune response compared with those in the control group, suggestive of oxidative stress caused by ezetimibe treatment.

Pathway analysis of the metabolic network showed unique metabolic changes in the ezetimibe group compared with the control group. In the control group, genes involved in the CoA-biosynthesis pathway were coordinately downregulated, and those in the glycerol-3 phosphate pathway coordinately upregulated, suggesting activated triacylglycerols biosynthesis. In the ezetimibe group,

Table 6 Changes in hepatic fatty acid composition

Fatty acid	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
C12:0 (lauric acid)	7.7±1.2	15.2±5.6	0.219	6.3±1.8	18.8±4.7	0.019	0.494
C14:0 (myristic acid)	19.9±2.5	33.0±10.1	0.228	17.6±2.2	56.6±13.0	0.014	0.148
C16:0 (palmitic acid)	185.9±23.8	303.9±118.2	0.334	169.7±22.9	583.9±176.8	0.042	0.202
C16:1n-7 (palmitoleic acid)	24.2±4.5	37.3±13.4	0.362	22.3±4.3	51.9±13.2	0.031	0.368
C17:0 (margaric acid)	4.6±0.7	3.5±0.1	0.400	5.3±0.8	16.0±4.1	0.024	0.025
C18:0 (stearic acid)	45.9±4.4	54.4±8.9	0.283	56.0±7.1	125.1±30.2	0.017	0.042
C18:1n-9 (oleic acid)	166.4±25.1	250.2±91.6	0.367	173.9±30.6	381.9±84.3	0.017	0.288
C18:2n-6 (linoleic acid)	80.4±12.3	87.9±22.5	0.556	73.9±8.5	147.3±36.1	0.035	0.066
C18:3n-6 (γ-linolenic acid)	ND	ND		ND	ND		
C18:3n-3 (α-linolenic acid)	0.6±0.4	0.0±0.0	0.171	0.6±0.4	0.0±0.0	0.178	0.981
C20:0n-6 (arachidic acid)	ND	ND		ND	ND		
C20:1n-9 (eicosenoic acid)	5.5±1.1	4.7±1.9	0.639	5.7±1.0	13.1±4.8	0.170	0.168
C20:2n-6 (eicosadienoic acid)	ND	ND		ND	ND		
C20:3n-6 (dihomo-γ-linolenic acid)	ND	ND		ND	ND		
C20:3n-9 (eicosatrienoic acid)	ND	ND		ND	ND		
C20:4n-6 (arachidonic acid)	ND	ND		ND	ND		
C20:5n-3 (eicosapentaenoic acid)	ND	ND		ND	ND		
C22:0 (behenic acid)	ND	ND		ND	ND		
C22:1n-9 (erucic acid)	14.2±2.5	11.7±2.7	0.474	16.2±2.4	19.2±1.0	0.664	0.468
C22:2n-6 (docosadienoic acid)	2.8±1.0	1.8±1.0	0.433	22.3±0.7	62.3±2.9	0.176	0.152
C22:4n-6 (docosatetraenoic acid)	ND	ND		ND	ND		
C22:5n-3 (docosapentaenoic acid)	ND	ND		ND	ND		
C22:6n-3 (docosahexaenoic acid)	13.6±3.5	7.8±3.3	0.232	14.2±3.7	48.7±19.9	0.109	0.097
C24:1 (nervonic acid)	ND	ND		ND	ND		

The data are expressed as 10^{-4} mg/mg liver, means ± SE

[a] p value for the intragroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

ND, not determined

genes involved in the L-carnitine pathway, including *CPT1A,* were coordinately downregulated. A decreased L-carnitine pathway could be associated with reduced β-oxidation of palmitic acids in mitochondria, resulting in an increase in long-chain fatty acids (lauric, myristic, palmitic, palmitoleic, margaric, stearic, oleic and linoleic acids). Unbalanced fatty acid composition could induce oxidative stress and lead to insulin resistance in the ezetimibe group. In addition, genes involved in the cholesterol and NEFA biosynthesis, including *SREBF2*, were coordinately upregulated in the ezetimibe group (Table 3), probably as a result of deceased absorption of exogenous cholesterol. Upregulation of *SREBF2* potentially represses the expression of hepatocyte nuclear factor 4, which is required for *CPT1* transcription [35]. Moreover, recent reports have demonstrated that microRNA (miR)-33, encoded by an intron of *Srebp2* [36], inhibits translation of transcripts involved in fatty acid β-oxidation, including *CPT1* [37]. miR-33 is also implicated in decreased insulin signalling by reducing insulin receptor substrate-2 [38, 39]. Hepatic gene expression profiles may, to some extent, explain hepatic fatty acid composition and impaired glycaemic control in the ezetimibe group. These novel SREBP-2-mediated pathways in the gene expression network may be relevant to a recent report that a polymorphism in the *SREBF2* predicts incidence and the severity NAFLD and the associated glucose and lipid dysmetabolism [15]. These unique hypotheses should be confirmed in future in vitro and in vivo studies.

Our study has some limitations. First, the number of patients is relatively small because the data and safety monitoring board recommended that the study intervention and enrolment be discontinued in light of the higher proportion of adverse events in the ezetimibe group than in the control group. Second, our trial was a 6 month open-label study that resulted in subtle changes in liver pathology compared with previous reports [40]. Indeed, a 6 month duration may be too short a period to expect improvement of fibrosis, which is a slowly

progressive process [40]. Third, the average serum aminotransferase levels were lower than those in previous studies [9, 10], and most of the patients had mild steatosis, fibrosis and lower NAS at baseline before ezetimibe treatment. Serum ALT levels did not decrease with ezetimibe treatment in the present study, in contrast to the significant improvement reported previously [9, 10]. And finally, secondary outcomes are always at risk of false-positive associations. Therefore, we not only presented the changes in HbA_{1c} ($p=0.001$ for ezetimibe treatment and $p=0.041$ for the intergroup difference at the end of the study), but also showed the signature of hepatic fatty acid composition and hepatic gene expression profiles that support the hypothesis that ezetimibe increases HbA_{1c} and hepatic fatty acids contents possibly through the SREBP-2–miR33 pathway. No previous studies have raised this issue, which is worth investigating. The same mechanism may underlie a statin-induced deterioration of glucose tolerance, which remains a serious concern. Furthermore, the SREBP-2–miR33 pathway may raise a concern for a safety issue of combination therapy with ezetimibe and statins because these agents may additively upregulate *SREBF2* expression [41]. Future large-scale, long-duration studies involving more severely affected patients are required to determine the definite efficacy and risks of ezetimibe in the treatment of NAFLD.

In conclusion, the present study represents the first randomised controlled clinical trial of the efficacy of ezetimibe on liver pathology, energy homeostasis, hepatic fatty acid composition and hepatic gene expression profiles in patients with NAFLD. The lipid profile and liver histology of cell ballooning and fibrosis were significantly improved by ezetimibe treatment. However, our findings suggest an increase in oxidative stress, insulin resistance and HbA_{1c} on treatment with ezetimibe, which should be taken into consideration in NAFLD patients.

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Contribution statement YT designed the study, recruited the patients, analysed the data and wrote the manuscript. TT designed the study, recruited the patients, interpreted the data and edited the manuscript. MH analysed the hepatic gene expression profiles. YK performed the statistical analyses. YZ analysed all the biopsies. KK, HM and TO recruited the patients and collected the clinical information. HS, KA and TY performed the liver biopsies and histological examinations. MN performed the DNA chip experiments. KY and EM analysed the hepatic fatty acid compositions. SK initiated and organised the study. All authors contributed to the acquisition, analysis and interpretation of data and the drafting and editing of the manuscript. All of the authors approved the final version of the manuscript.

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