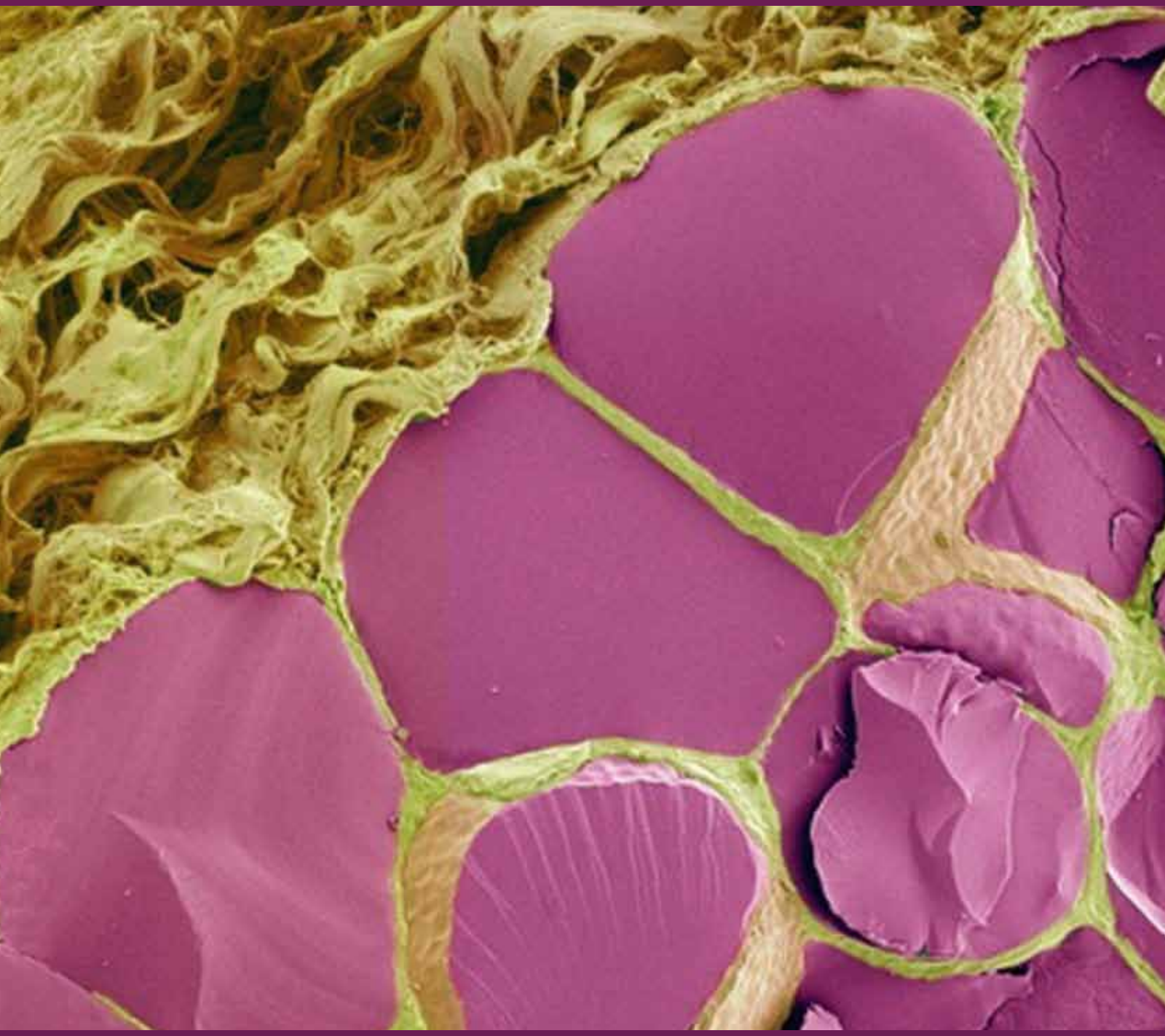
International Journal of Endocrinology

Nonalcoholic Fatty Liver Disease: Its Mechanisms and Complications

Guest Editors: Abdelfattah El Ouaamari and Kaori Minehira



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Editorial

Nonalcoholic Fatty Liver Disease: Its Mechanisms and Complications

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Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent disorder in which fat accumulates excessively in the liver. The disease affects between 10% to 30% of the general population and up to 90% in obese patients [1]. Fatty liver, the first step of NAFLD, is linked with insulin resistance, a well-known condition that predisposes to the development of metabolic disorders such as diabetes and obesity. Although NAFLD may be asymptomatic, the development of nonalcoholic steatohepatitis (NASH) results in hepatic fibrosis, cirrhosis, and ultimately might evolve to liver cancer. While substantial progress has been made in understanding the disease, the mechanisms governing the development of hepatic steatosis and fibrosis are still unclear, and their effects on metabolism need to be clearly understood.

NAFLD has been associated with metabolic disorders, and many confounders such as hyperlipidemia, hypertension, diabetes, and obesity make research in the field very difficult to conduct. To understand the impact of NAFLD on the development of cardiovascular disease (CVD), H. Lu et al. performed a meta-analysis on 4 cross-sectional and 2 prospective cohort studies in European and Asian populations. After adjustments for confounders (age, sex, HbA1c, plasma lipid and liver enzyme levels, and metabolic syndromes), NAFLD significantly predicted CVD suggesting the prime role of the liver steatosis on the development of metabolic syndrome.

The association between NAFLD and metabolic diseases has also been reported in children [2]. Here, L. Pacifico et al. further investigated the association between NAFLD and thyroid function in childhood obesity. In this Italian cohort of 402 overweight/obese children (age 10–12 years old), eighty-eight children (21.9%) had TSH above the normal range (>4.0 mIU/L). High TSH was associated with many metabolic variables such as hepatic steatosis, hypertriglyceridemia, elevated total cholesterol, and insulin resistance after adjustment for age, gender, pubertal status, and BMI. Stepwise multivariate regression analysis showed a significant association between hyperthyrotropinemia and hepatic steatosis (OR = 1.96). Other covariates independently associated with hepatic steatosis were hypertriglyceridemia (OR = 2.73) and insulin resistance (OR = 2.37). This study clearly demonstrated the complexity of metabolic disorders even in children. Given the growing population in obese children, it is important to understand how metabolic comorbidities develop in children in order to prevent earlier metabolic derangements.

A review from H. Rodríguez-Hernández et al. "Obesity and Inflammation: Epidemiology, Risk Factors, and Markers of Inflammation" tries to understand the causal role of low-grade inflammation on the development of different metabolic comorbidities such as CVD, diabetes, and metabolic syndrome in obese conditions. Authors concluded that abnormal levels of proinflammatory cytokines such as C-reactive protein, tumor-necrosis factor-α, and interleukine-6 play a central role in obesity and are strongly associated with increased risks of metabolic syndrome. These cytokines are originated from macrophage residing at adipose tissues. Obesity therefore mediates the inflammatory circumstance via increased adipose mass and leads to many metabolic disorders, such as CVD, NAFLD, and type 2 diabetes.

Another factor secreted from adipose tissue is nonesterified fatty acids (NEFA) via lipolysis. Increased fasting NEFA has been thought to induce insulin resistance and NAFLD. However, recent study on nonoxidative fatty acid disposal (fatty acids being used for VLDL or intracellular lipids synthesis) reported no correlation with whole body insulin sensitivity in obese women [3]. Therefore, F. M. Finucane et al. tried to elucidate the association between NEFA ("fasting" versus "postprandial") and insulin sensitivity after an oral glucose loading in the Hertfordshire Physical Activity Trial (healthy elderly males). They provided evidence that impaired postprandial NEFA suppression, but not fasting NEFA, contributes to the association between whole body insulin resistance. This paper also indicated that the impaired suppression of NEFA was directly associated with increased intrahepatic lipid in elderly.

One paper in this special issue studied the role of inflammatory pathways on the development of hepatic fibrosis. G. Willemin et al. revisited the proportional contribution of major histocompatibility class II (MHCII) molecules to the development of liver diseases. Using a model of a complete disruption of MHCII pathway, they demonstrated that in contrast to the traditional thoughts MHCII signaling was not critical to the development of steatosis-induced inflammation or fibrosis. This conclusion was build up on experiments where animals lacking MHCII challenged with high-fat diet or carbon-tetra-chloride (CCl_4)-induced hepatic cirrhosis did develop NASH and fibrosis, respectively, at the same extent as in littermate controls. Although SNPs on MHCII genes were reported to increase a risk of hepatic inflammation and fibrosis, this study demonstrated that MHC II pathway was not required in the development of NAFLD in mice.

Three papers in this issue investigated the influence of diet or dietary supplement on hepatic steatosis. C. Gonzalez et al. reported a clinical study in which they assessed the influence of dietary intake (carbohydrate, lipid, protein, and energy) on hepatic steatosis and fibrosis in patients with NAFLD. Authors found that energy and carbohydrate intake were positively correlated with liver steatosis but not with fibrosis. Authors suggested that even moderate reductions of dietary carbohydrate might help to reduce liver fat in NAFLD. C. F. Jin et al. studied the effect of Eucommia Ulmoides Oliver cortex extracts (EUCE), a Chinese herbal extract, in the development of NAFLD induced by CCl_4. Mice acutely treated by CCl_4 developed hepatic steatosis and induced endoplasmic reticulum (ER) stress and oxidative stress. Pretreatment by EUCE dose dependently reduced the development of steatosis via a normalization of ApoB secretion and a reduction in ER stress, and malondialdehyde. Authors concluded that EUCE might protect liver against CCl_4-induced hepatic lipid accumulation, ER stress and its related ROS dysregulation. This study was conducted in the case of CCl_4-induced hepatic steatosis; it would be interesting to investigate the effect of EUCE on high-fat high-sugar diet-induced hepatic steatosis and hepatic insulin resistance. In the review article, A. B. Ross et al. emphasize the beneficial effect of whole grains versus refined grains in the prevention and treatment of NAFLD. Whole grains have higher content in many nutrients and phytochemicals than their refined counterparts. Authors proposed several possible mechanisms to improve NAFLD via increased whole grains intake: (i) reduction of total energy intake, (ii) changes to and stimulation of gut microbiota, and (iii) specific actions of phytochemicals (e.g., vitamins, phenolic acids, and betaine). This review provides us the most current information on how whole grains could impact on NAFLD.

This special issue is dedicated to review articles, research articles and clinical studies focused on timely topics concerning liver steatosis in the context of inflammation, and how the disease relates to other metabolic disorders and the impact of diet on the development and the prevention and/or treatment of NAFLD. Divers articles in this special issue highlight broad interactions between NAFLD and metabolic disorders and its complexity. And it is evident that additional efforts are needed to gain greater insights into the mechanisms underlying the development of liver steatosis, NASH, and cirrhosis. Gained knowledge will provide opportunities for effective medications/diets for the treatment/prevention of NAFLD and associated complications.

We hope that the information published in this special issue enriches the knowledge of our readers and scholars interested in general in the field of liver diseases and in particular in NAFLD.

Abdelfattah El Ouaamari
Kaori Minehira

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Research Article

Mechanism of the Inhibitory Effects of *Eucommia ulmoides* Oliv. Cortex Extracts (EUCE) in the CCl_4-Induced Acute Liver Lipid Accumulation in Rats

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Eucommia ulmoides Oliv. (EU) has been used for treatment of liver diseases. The protective effects of *Eucommia Ulmoides* Oliv. cortex extracts (EUCE) on the carbon tetrachloride- (CCl_4-) induced hepatic lipid accumulation were examined in this study. Rats were orally treated with EUCE in different doses prior to an intraperitoneal injection of 1 mg/kg CCl_4. Acute injection of CCl_4 decreased plasma triglyceride but increased hepatic triglyceride and cholesterol as compared to control rats. On the other hand, the pretreatment with EUCE diminished these effects at a dose-dependent manner. CCl_4 treatment decreased glutathione (GSH) and increased malondialdehyde (MDA) accompanied by activated P450 2E1. The pretreatment with EUCE significantly improved these deleterious effects of CCl_4. CCl_4 treatment increased P450 2E1 activation and ApoB accumulation. Pretreatment with EUCE reversed these effects. ER stress response was significantly increased by CCl_4, which was inhibited by EUCE. One of the possible ER stress regulatory mechanisms, lysosomal activity, was examined. CCl_4 reduced lysosomal enzymes that were reversed with the EUCE. The results indicate that oral pretreatment with EUCE may protect liver against CCl_4-induced hepatic lipid accumulation. ER stress and its related ROS regulation are suggested as a possible mechanism in the antidyslipidemic effect of EUCE.

1. Introduction

Eucommia ulmoides Oliv. (EU) is one of the most popular tonic herbs in Asia. In a traditional herbal prescription, EU is used either as a single herb or in combination with one or two of the other herbs [1]. EU is also a popular folk drink and is used as a functional food reinforcing the muscles and lungs, lowering blood pressure, preventing miscarriages, improving liver and kidney tone, and increasing longevity [2]. EU, prepared from leaves or bark, is commonly used as liver and kidney tonic, thus, improving detoxification and circulation by the liver [3] and kidney [4, 5], respectively. EU leaves have been used for treatment of hepatic lipid accumulation [6, 7] and hepatic damage [3]. Recently, it was reported that *Eucommia ulmoides* Oliv. cortex extracts (EUCE) contain the same components as EU leaves, which have been the focus of medical research [2]. Studies have shown that EU leaf extracts have potent protective effects in various lipid peroxidation models and reduce oxidative damage of biomolecules [3, 8–11].

Hepatic accumulation of triglyceride (steatosis) is a major complication associated with obesity, insulin resistance, and alcoholic and nonalcoholic fatty liver disease [12]. This is because of increasing lipogenesis and decreasing β-oxidation followed by lipid peroxidation and mitochondrial dysfunction [12]. If left untreated, benign steatosis can develop into steatohepatitis, fibrosis, or cirrhosis.

Carbon tetrachloride (CCl_4) is a colorless liquid that was commonly used as an anesthetic in the 19th and early 20th century. However, CCl_4 was banded after establishment of

its hepatotoxicity in the first 25 years of the 20th century. CCl_4-induced liver damage in rats is the best characterized animal model of xenobiotic-induced free radical-mediated liver diseases [13]. Depending on the dose and duration, the effects of CCl_4 on hepatocytes are manifested histologically as hepatic steatosis, fibrosis, hepatocellular death, or carcinogenicity [14]. Triglyceride secretion depends on the function of endoplasmic reticulum (ER) which assembles and secrets apolipoproteins in the liver. If ER function is damaged, secretion of apolipoproteins such as apolipoprotein B (ApoB) is inhibited, leading to hepatic lipid accumulation [15]. After oral administration, CCl_4 concentrates in the liver, resulting in rapid accumulation of triglycerides in the liver [15]. Recently, CCl_4 was shown to induce reactive oxygen species (ROS) through activation of cytochrome P450, leading to ER stress-mediated dysfunction. CCl_4 is transformed to trichloromethyl free radical ($CCl_3OO^{\bullet}$) by cytochrome P450 enzymes. Specifically, P450 2E1 interacts with NADPH-dependent cytochrome P450 reductase (NPR). Electron uncoupling between NPR and P450 2E1 is a major source of ROS on the ER membrane. ROS attack polyunsaturated fatty acid portions of membrane lipids to propagate a chain reaction, leading to lipid peroxidation and disruption protein synthesis, which results in the accumulation of proteins in the ER lumen and induction of ER stress [16]. It has been reported that severe and prolonged ER stress causes the accumulation of free radicals and disruption of protein secretion, leading to alteration of pathological conditions [17]. Regulation of ER stress has been suggested as one of the therapeutic/preventive approaches for the treatment of pathological conditions/diseases with ER stress [18]. Lysosomes are membrane-enclosed organelles that contain acid hydrolase enzymes. Lysosomal enzymes are known to play a role in regulating the ER stress response [19]. Proteins accumulated during ER stress are degraded by lysosomal enzymes through the endoplasmic reticulum-associated degradation (ERAD) pathway [20]. The lysosome-induced ERAD pathway has been suggested as one of the regulatory mechanisms of ER stress because the lysosomal activation can relieve intra-ER unfolded protein folding requirement.

ER stress regulation can be one of the potential mechanisms for ROS-associated hepatic steatosis. The secretion of ApoB is also altered in the presence of ER stress [21, 22]. CCl_4-induced steatosis is related to ER stress and its related dysfunctions such as the alteration of apolipoproteins and ROS accumulation. Accordingly, the aim of this study is to investigate the effect of EUCE in CCl_4-induced hepatic steatosis and ER stress. This study suggests that the preventive/therapeutic effect of the cortex extracts is due to the regulation of ER stress through lysosomal activation.

2. Materials and Methods

2.1. Materials. *Eucommia ulmoides* Oliv. cortex was purchased from Sam-Hong Company (Seoul, Korea). Carbon tetrachloride (CCl_4) and oil red O were purchased from Sigma-Aldrich Company (MO, USA).

2.2. Preparation of Plant Extracts. Dried cortex of *Eucommia ulmoides* Oliv. was authenticated in the Department of Pharmaceutical Chemistry of Yonsei University, Korea. The cortex was ground into a powder, mixed with extraction solvent (25% ethanol) at the ratio of 1 : 12, and then incubated in a 70°C water bath for 2 hours. Following incubation, the extraction solution was filtered, evaporated, and then dried to a powder by freeze drying at −55°C under low pressure.

2.3. Treatment of Animals. Forty-eight Sprague Dawley (SD) male rats weighing 240–250 g (8-week-old) were purchased from Samtako Inc. (Osan, Korea) and housed in an air-conditioned room at 22 ± 2°C with a 12 h light/dark cycle. Animals were fed with rodent chow and tap water *ad libitum*. To study the protective effect against the CCl_4-induced acute liver lipid accumulation, all rats were randomly divided into six groups of eight rats each: (A) control group, (B) CCl_4 group, (C) CCl_4 + EUCE 0.25 g/kg, (D) CCl_4 + EUCE 0.5 g/kg, (E) CCl_4 + EUCE 1 g/kg, and (F) EUCE 1 g/kg. Rats of groups B, C, D, and E were intraperitoneally injected with 1 mg/kg CCl_4 mixed in olive oil, and rats of groups A and F were intraperitoneally injected with the same volume of pure olive oil. Rats in groups C, D, E, and F were treated with EUCE 8 times (twice/day for four days) before the injection of CCl_4 (except for rats in group F). Four hours after CCl_4 injection, each rat was anesthetized, blood was drawn, and liver tissues were removed. Blood samples were collected for ALT, AST, TG, and TC assays. Livers were excised from the animals and assayed for GSH levels, MDA formation, and pathological histology, according to the procedures described below. All experimental procedures were conducted in accordance with the National Institutes of Health. This experiment was approved by the Institutional Animal Care and Use Committee of Chonbuk National University, Jeonju, Korea.

2.4. Histological Staining. Liver samples were fixed in 3.7% formalin and dehydrated with 20% and 30% sucrose. Then, liver samples were embedded in OCT compound and cut into 10 μm sections for oil red O staining. Liver sections were fixed in 3.7% formalin for 5 minutes washed with 60% isopropanol. Fixed samples were then stained with 0.3% oil red O in 60% isopropanol for 30 min and washed with 60% isopropanol. Sections were counterstained with hematoxylin, washed with running water for 5 min, and mounted with an aqueous solution. Stained sections were quantified by histomorphometry.

2.5. DPPH Radical Scavenging Assay. Free radical scavenging activity of the EUCE was measured using the 1, 1-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [23]. EUCE solution (0.3 mL) at a range of concentrations was mixed with 0.2 mM DPPH in methanol (2.7 mL). The mixture was shaken vigorously and allowed to stand for 1 h before the absorbance was measured at 517 nm. Free radical scavenging activity was calculated as the following percentage: $[(A_s - A_i)/A_s] \times 100$ (A_s is absorbance of DPPH alone and A_i is absorbance of DPPH in the presence of various extracts). Butylated hydroxyl toluene (BHT) at a concentration identical to the experimental samples was used as a reference.

2.6. Biochemical Determination. Serum levels of triglyceride (TG), total cholesterol (TC), alanine transaminase (ALT), aspartate aminotransferase (AST), liver glutathione (GSH), and malondialdehyde (MDA) were determined using a commercial analysis kit obtained from the ASAN Institute of Biotechnology (Seoul, Korea) and Jiancheng Institute of Biotechnology (Nanjing, China). Hepatic concentrations of TC and TG were also measured after chloroform-methanol extraction. Liver samples (115 mg) mixed with 500 μL D-PBS were homogenized and centrifuged at 3500 g for 5 minutes. The supernatants were removed and centrifuged briefly after the addition of 400 μL chloroform-methanol (1 : 2). Then, 250 μL chloroform and 250 μL water were added, and the samples were centrifuged at 3000 rpm for 5 minutes. The lower phase was transferred to a new tube, and residual chloroform was evaporated by heating at 55°C. After chloroform evaporation, 25 μL of RIPA buffer was added and the samples were resuspended by heating at 90°C for 3 minutes. TG and TC levels were then measured with commercial kits.

2.7. Western Blot Analysis. Proteins were separated under nonreducing conditions, transferred to nitrocellulose membranes, and incubated for 2 h at room temperature in blocking buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk). Blots were washed three times and incubated overnight at 4°C in the same buffer containing 0.5% dry milk and primary antibody (1 : 1000 dilution). The blots were then washed and incubated with mouse horseradish peroxidase-conjugated secondary antibody (1 : 4000) in 1.0% skim milk for 1 hour at room temperature. Immune reactivity was detected by chemiluminescence. Then, the intensities of band were measured and quantified as described by Luke Miller (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/).

2.8. Measurement of P450 2E1 Activity. Specific activity for P450 2E1 was evaluated in liver homogenates utilizing model substrates. P450 2E1 catalyzes the hydroxylation of *p*-nitrophenol to *p*-nitrocatechol [24]. First, we isolated liver microsomes. Then, microsomal proteins were incubated in assay buffer (1 mM ascorbic acid, 2 mM magnesium chloride, 1 mM NADPH, and 50 mM phosphate buffer, pH 6.8) at 37°C in a shaking water bath. Following incubation, the P450 2E1 reaction was stopped on ice with addition of 20% trichloroacetic acid. Samples were concentrated to 50 μL, and then the supernatants were transferred to a 96-well plate. Prior to reading, 2 M NaOH was added to each sample or standard. Absorbance was measured at $\lambda = 517$ nm on a 96-well plate reader [25].

2.9. Measurement of Lysosomal Enzymes Activity. Lysosomes were isolated from liver tissues for the measurement of lysosomal enzymes activity. Isolation of lysosomes was performed using a method based on differential and density-gradient centrifugation techniques [26]. After isolation of lysosomes, the 100 μL assay mixtures consisted of the following: β-galactosidase assay, 0.5 mM 4-methylumbelliferyl- (MU-) β-galactoside in 100 mM citrate-phosphate buffer (pH 4.35) with 0.4 M NaCl; β-glucuronidase assay, 1 mM 4-β-glucuronide in 100 mM acetate buffer (pH 4.0); α-mannosidase assay, and 4 mM α-mannopyranoside in 100 mM acetate-phosphate buffer (pH 4.0). Assay mixtures were incubated at 37°C for 30 minutes with an excitation wave length from 360 nm to 410 nm.

2.10. Statistical Analysis. All data are expressed as mean ± SD. All comparisons were done by one-way analysis of variance (ANOVA) with Dunn's test for post hoc analysis. P values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of EUCE on CCl_4-Induced Histological Changes in the Liver. Liver tissues were collected to assess the effect of EUCE on liver pathological changes. As shown in Figure 1(a), H&E staining of liver sections demonstrated normal liver architecture in CCl_4-treated rats. Oil red O staining showed the development of acute lipid accumulation in rats 4 hours after injection of CCl_4, whereas no histological abnormalities were observed in normal control or EUCE-treated rats (Figure 1(b)). Administration of EUCE prevented fatty deposition in hepatocytes. As demonstrated by histological results, H&E staining indicated no morphology changes in the CCl_4-treated rats compared with the control group, while oil red O staining showed that CCl_4-induced lipid accumulation was blocked by pretreatment with EUCE. Specifically, a significantly reduced in lipid accumulation was observed with administration of EUCE at a dose of 1 g/kg.

3.2. Effect of EUCE on Lipid Metabolism. To analyze the possible role of EUCE in lipid metabolism, which plays a major role in fatty liver formation, triglyceride (TG) and total cholesterol (TC) in liver and serum were investigated. As shown in Figure 2, TG and TC levels were significantly increased by acute CCl_4 injection, and this was blunted by EUCE pretreatment. The data suggested that EUCE may regulate acute lipid accumulation.

3.3. Effect of EUCE on Free Radical Scavenging Activity and Hepatic GSH Levels. The effect of EUCE on DPPH free radical scavenging activity was tested, and the results are presented in Figure 3(a). As shown in Figure 3(a), IC_{50} values of EUCE were about 310 μg/mL. EUCE exhibited a curve of antioxidant activity. Glutathione (GSH) constitutes the first line of defense against free radicals [27]. GSH levels were significantly depleted by CCl_4 administration; however, depletion of GSH induced by CCl_4 was significantly reversed in a dose-dependent manner by pretreatment with EUCE (Figure 3(b)).

3.4. Effect of EUCE on CCl_4-Induced Lipid Peroxidation Levels. CCl_4-induced ROS accumulation has been associated with the pathology status induced by CCl_4 [28]. We investigated MDA content which is a result of lipid peroxidation by ROS.

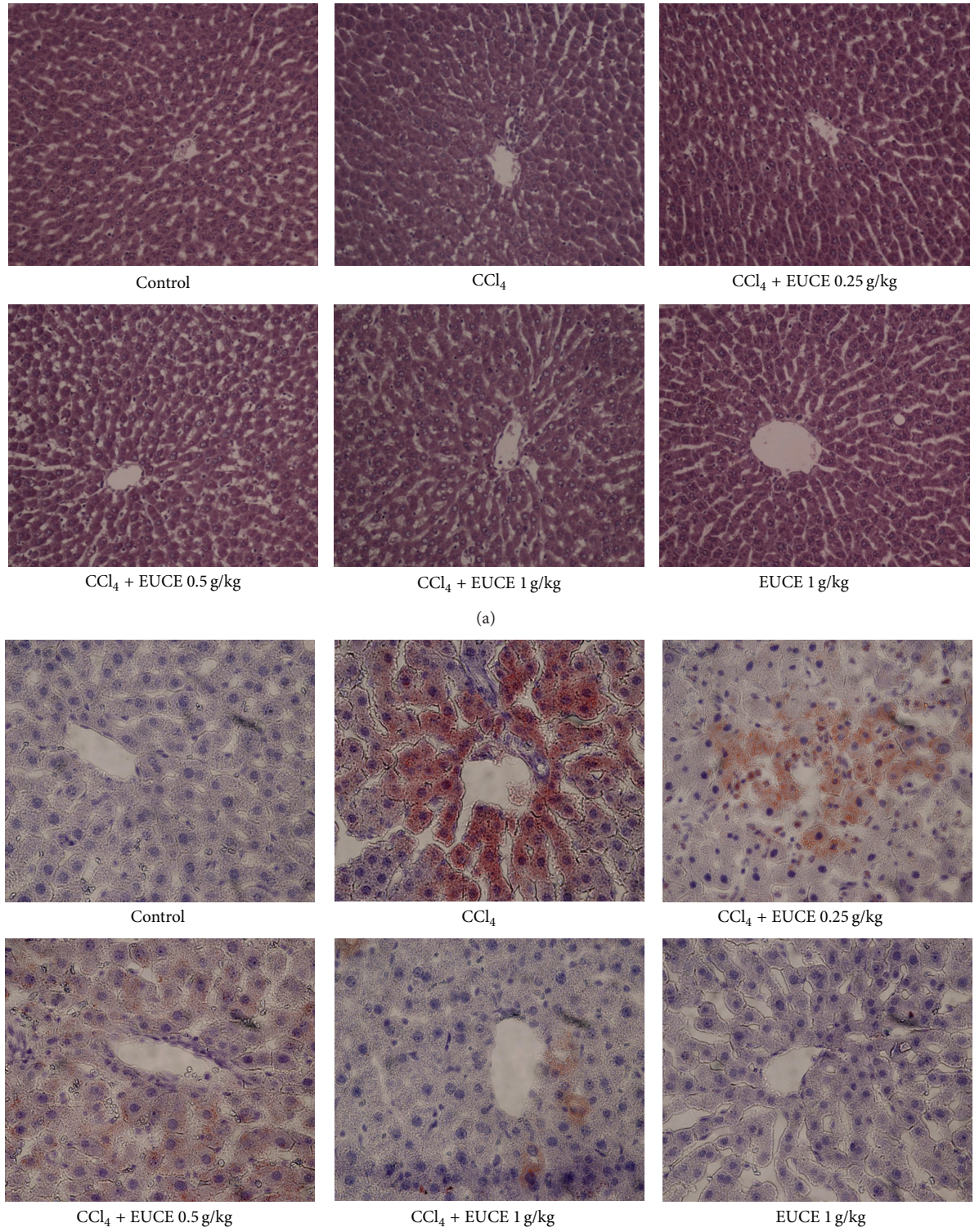

Figure 1: Effect of EUCE on CCl_4-induced histological changes in liver. Rats were injected with 1 mg/kg CCl_4, and livers were isolated after 4 hours. Representative photomicrographs (200x) of liver sections from rats ($n = 8$) stained with hematoxylin and eosin (a) and oil red O (b) are shown.

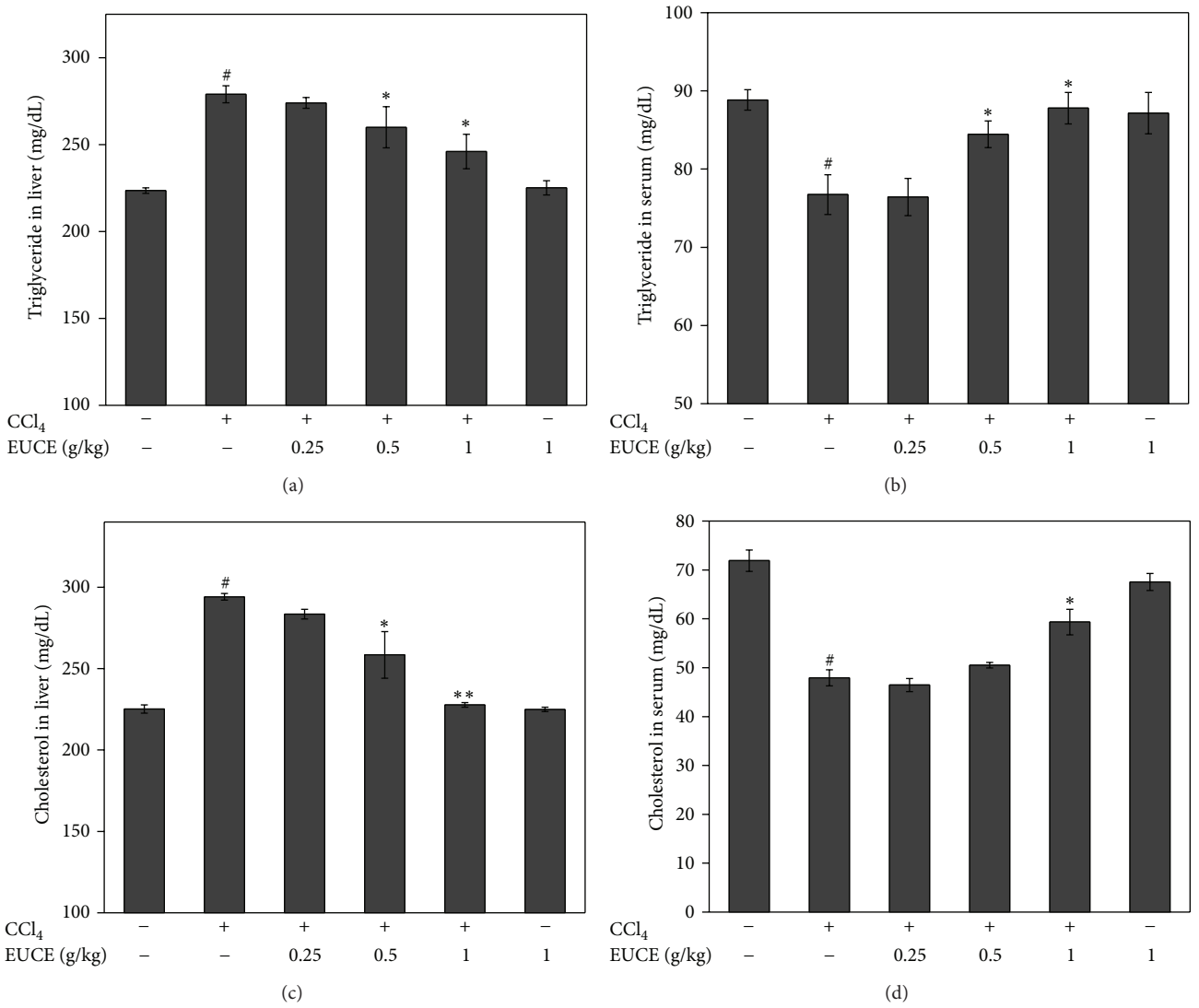

Figure 2: Effect of EUCE on CCl_4-induced inhibition of hepatic triglyceride and cholesterol secretion. Rats were injected with 1 mg/kg CCl_4, and livers were isolated after 4 hours. Triglyceride and cholesterol levels were measured in the liver (a), (c) and plasma (b), (d), respectively. Values are mean ± SD, $n = 8$. Asterisks indicate differences from the group treated with CCl_4 only ($^*P < 0.05$; $^{**}P < 0.001$). $^{\#}P < 0.05$ indicates a significant difference compared with the control group.

Compared with the control group, the CCl_4-treated group showed significantly increased MDA content (Figure 3(c)). However, EUCE treatment at dose of 0.5 and 1.0 g/kg significantly decreased MDA content. These results indicate that EUCE has the potential to reduce lipid peroxidation induced by CCl_4. Furthermore, ALT and AST, hepatic enzymes that are released into the blood stream by liver damage, were not increased (Figures 3(d) and 3(e)). Thus, liver function was not affected during the transient time periods.

3.5. Effect of EUCE on ApoB and ApoA1 Levels in the Liver. Lipids are carried on apolipoproteins (Apo) in plasma [29]. ApoA1 is responsible for carrying HDL, and ApoB is responsible for carrying LDL and triglyceride [30]. As shown in Figure 4, the expression of ApoB was increased in the liver after 4 hr of exposure to CCl_4, but ApoA1 was no affected. Increased expression of ApoB in the liver was suppressed by pretreatment with EUCE in a dose-dependent manner, particularly at a dose of 1 g/kg. Transient accumulation of triglyceride and cholesterol in liver induced by CCl_4 occurred via decreased plasma ApoB production and VLDL secretion [15]. These results show that pretreatment with EUCE may improve ApoB secretion compared with CCl_4-treated group.

3.6. Effect of EUCE on CCl_4-Induced ER Stress and P450 2E1 Activation. Abnormal ER function affects secretion of apolipoproteins. A previous study reported on the identification of proteins that play an important role in the survival of liver cells after induction of ER stress by CCl_4 [31]. As shown in Figures 4(a) and 4(b) in CCl_4-treated rats, the expression of ER stress proteins GRP78, CHOP, IRE-1α, and spliced XBP-1 was increased, and eIF-2α was phosphorylated in liver

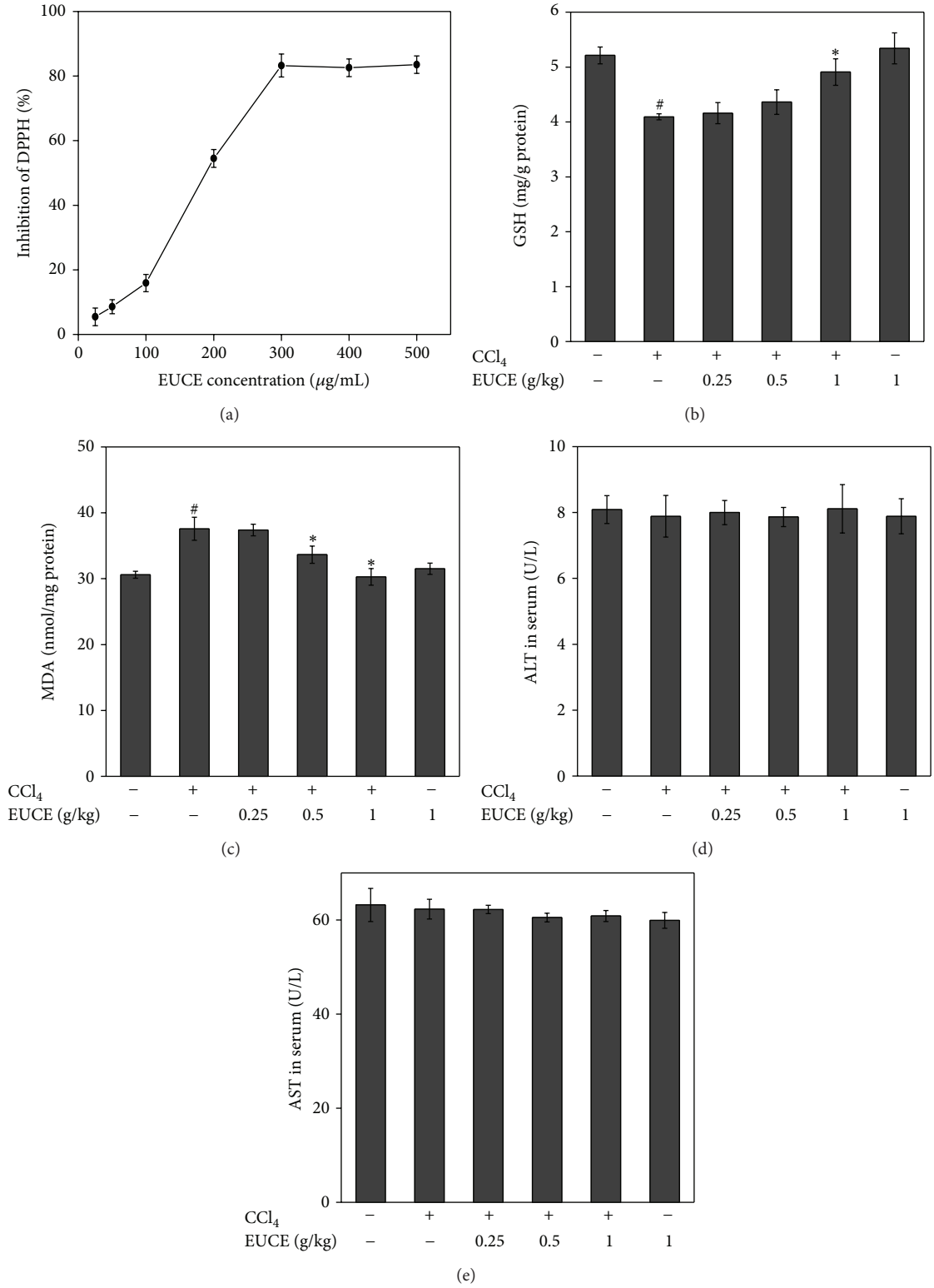

Figure 3: Effect of EUCE on free radical scavenging activity and CCl_4-induced increases in liver glutathione (GSH) and malondialdehyde (MDA). A difference was found in ALT and AST levels after injection of CCl_4. Values are mean ± SD, $n = 8$. Asterisks indicate differences from the group treated with CCl_4 only ($^*P < 0.05$). $^\#P < 0.05$ indicates a significant difference compared with the control group.

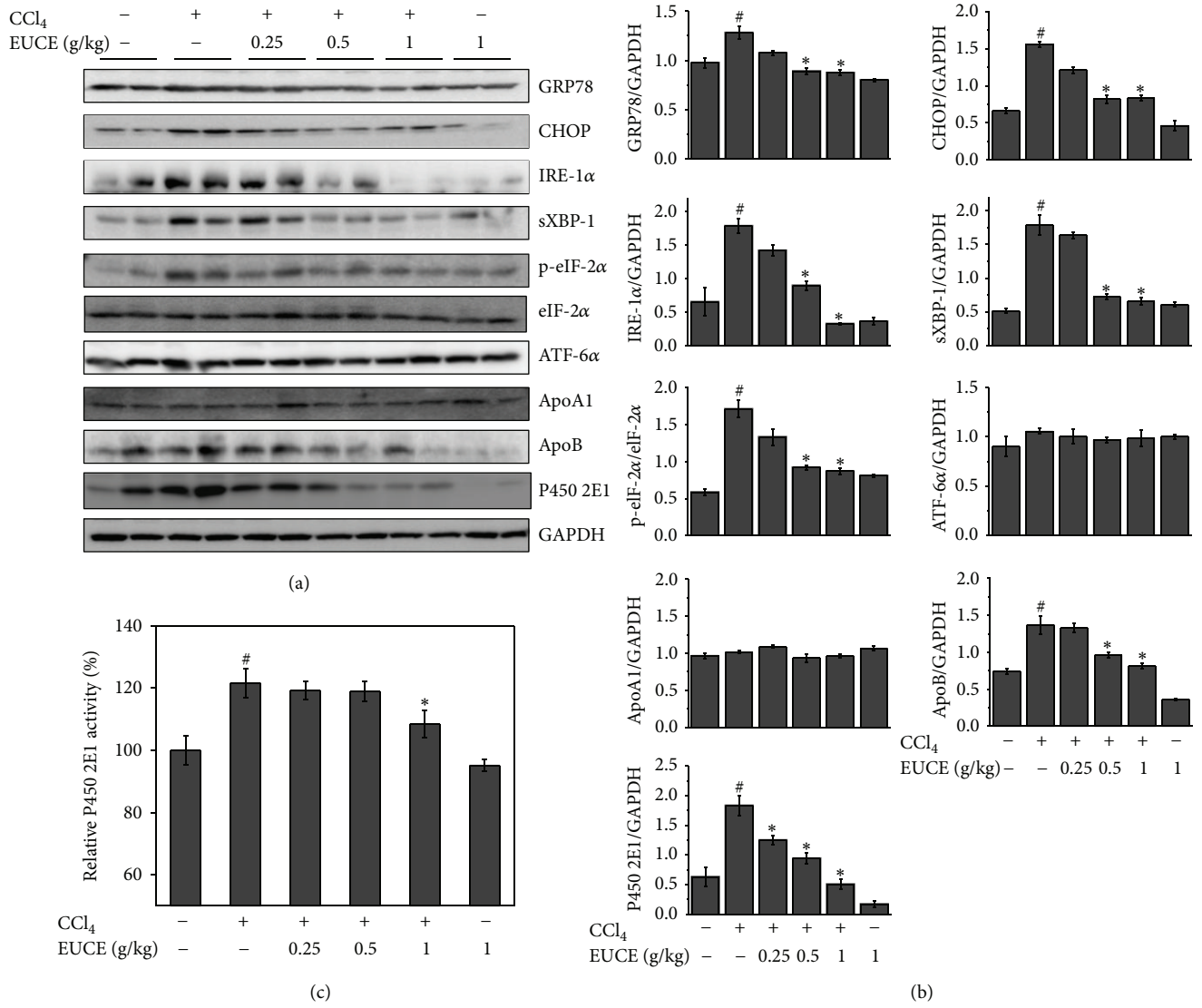

Figure 4: Effect of EUCE on CCl_4-induced ER stress response and expression of ApoB and P450 2E1. Rats were injected with 1 mg/kg CCl_4, and livers were isolated after 4 hours. (a) Western blotting was performed on liver protein extracts with anti-GRP78, CHOP, p-eIF-2α, eIF-2α, ATF-6α, IRE-1α, sXBP-1, ApoA1, ApoB, P450 2E1, or GAPDH. (b) Graphs showing quantification of (a). (c) P450 2E1 activity was measured in liver. Asterisks indicate differences from the group treated with CCl_4 only ($^*P < 0.05$). $^\#P < 0.05$ indicates a significant difference compared with the control group.

tissue. Pretreatment with EUCE reduced the expression of ER stress proteins in a dose-dependent manner. P450 2E1, the major isozyme involved in the bioactivation of CCl_4 and responsible for ER stress-induced ROS, was also increased in CCl_4-treated rats. Consistently, pretreatment of EUCE significantly reduced the P450 2E1 activity (Figure 4(c)).

3.7. Effect of EUCE on Lysosomal Enzyme Activity. Enhanced activity of lysosomal enzymes has been suggested to have a regulatory role on ER stress. Pretreatment with EUCE significantly increased the activity of lysosomal enzymes compared with the control group, particularly at a dose of 1 g/kg (Figure 5), thus, indicating the potential role of EUCE in enhancing lysosomal enzyme activity.

4. Discussion

This study showed that rats pretreated with EUCE were protected against CCl_4-induced hepatic lipid accumulation, as confirmed by histological observation and decreased levels of triglyceride and total cholesterol compared with the control group. EUCE regulated ER stress response by decreasing P450 2E1 activity and ROS accumulation. CCl_4-induced ER stress response, enhancing P450 2E1 activity. In our previous study, we showed that ER stress and its related P450 2E1 activity play an important role in CCl_4-induced steatosis in rats [32]. In the CCl_4-induced hepatic steatosis, possible role of ROS accumulation has been suggested, ER stress and its consequent increase of P450 2E1, leading to ROS accumulation. A converse mechanism, P450 2E1 activation

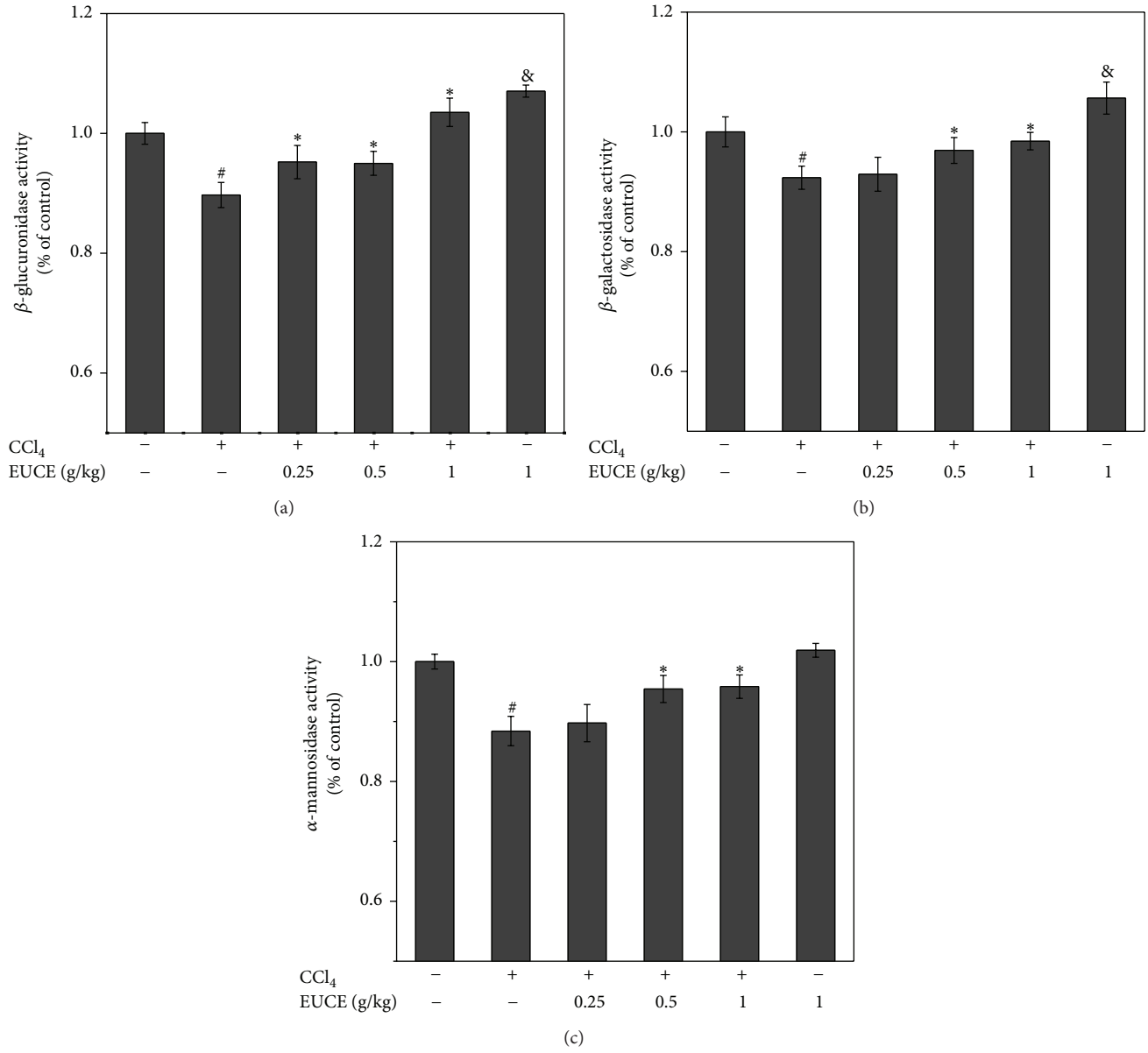

Figure 5: Effect of EUCE on CCl_4-induced lysosomal enzymes activity. Rats were injected with 1 mg/kg CCl_4, and livers were isolated after 4 hours. The lysosomal enzymes activity was measured by a kit. Values are mean ± SD, $n = 8$. Asterisks indicate differences from the group treated with CCl_4 only ($^{*}P < 0.05$). $^{\&}P < 0.05$, $^{\#}P < 0.05$ indicate a significant difference compared with the control group.

and ROS accumulation induced by CCl_4, leading to ER stress, is not able to be ruled out. Although the cause or consequence of ROS production in relation to ER stress has not been clearly established, the pieces of evidence of CCl_4-induced ROS production have been already accumulated. Recently, CCl_4 was shown to induce hepatotoxicity by enhancing the formation of free radicals through their metabolism, leading to lipid peroxidation of cellular and organelle membranes as a primary pathogenic step [33].

In this study, P450 2E1 activity was significantly increased 4 hours after exposure to CCl_4 compared with the control group, while the EUCE-pretreated group showed decreased P450 2E1 activity compared with the CCl_4-treated group (Figure 4(c)). CCl_4 is widely used for experimental induction of liver steatosis/cirrhosis in relatively acute settings [34]. Cytochrome P450 2E1, a member of the cytochrome P450 mixed-function oxidase system, can catalyze CCl_4 to form trichloromethyl free radicals which interact with molecular oxygen to form trichloromethyl peroxy radicals [16, 35–37]. These free radicals play an important role in the pathogenesis of liver steatosis by binding to proteins or lipids, which then initiates lipid peroxidation [38]. It has also been reported that P450 2E1 is the primary enzyme responsible for low-dose carbon tetrachloride metabolism in human liver microsomes

[39]. The hepatotoxic effects of CCl_4 are dependent on the cosubstrate NADPH because conversion of CCl_4 to $CCl_3OO^{\bullet}$ occurs in conjunction with the NADPH-cytochrome P450 electron transport chain in the liver endoplasmic reticulum [13, 40]. Cytochrome P450 transfers an electron from NADPH to CCl_4, causing CCl_4 to be reduced to $CCl_3OO^{\bullet}$ and $Cl^{\bullet}$. A previous study showed that the expression of P450 2E1 and its interaction with NPR both increase after CCl_4 treatment; however, both were also shown to decrease after 12 hours [32].

This study also showed that EUCE pretreatment increased GSH level that had been lowered by CCl_4. GSH helps prevent damage of important cellular components caused by reactive oxygen species such as free radicals and peroxides. Moreover, GSH plays a preventive/therapeutic role in CCl_4-induced hepatic toxicity via the P450 2E1 pathway [37]. GSH is an antioxidant that contributes to the detoxification of CCl_4, which induces hepatic lipid accumulation through its free radical derivatives [16]. As reported, increased production of ROS induced by CCl_4 plays a role in liver steatosis/cirrhosis through two distinct pathways. One pathway involving P450 2E1 leads to the formation of toxic peroxyl and alkoxyl radicals that initiate lipid peroxidation. The second pathway involves a detoxification reaction that lowers GSH levels [41, 42]. Therefore, the DPPH assay was used in this study to evaluate the effect of EUCE on free radical scavenging activity. EUCE may play an important role in raising GSH levels [43]. In addition, oxidative stress, which is considered to play an important role in the development of hepatic steatosis/cirrhosis, is associated with lipid peroxidation and lower levels of GSH [44].

In addition, the CCl_4-treated group had significantly increased levels of MDA, whereas the EUCE-pretreated group had significantly decreased levels of MDA in liver. MDA is a metabolite of the free radical-mediated lipid peroxidation cascade and therefore is used as a marker of lipid peroxidation. Thus, the biochemical mechanism underlying the development of CCl_4 steatosis/cirrhosis may involve MDA. In CCl_4-treated rats, significantly increased levels of MDA have been shown [45].

In this study, the expressions of ER stress proteins and hepatic ApoB were both increased in CCl_4-treated rats, whereas they were decreased in the EUCE groups (Figures 4(a) and 4(b)). This result suggests that EUCE might modify ApoB synthesis and therefore could impact on liver and plasma triglyceride content. Apolipoproteins, lipid binding proteins that form lipoproteins to transport lipids through the lymphatic and circulatory systems, are regulated by normal function of the ER. With ER stress, protein folding and secretion can be significantly affected [46]. As reported, ApoB, a member of the apolipoprotein family, is reduced in CCl_4-treated rats [47]. CCl_4 decreases secretion of very low density lipoproteins and rapidly increases triglycerides in rat livers [48]. Consistently, in CCl_4-treated groups, triglycerides rapidly accumulate in liver, contributing to the failure of secretory mechanisms [49]. It has been reported that decreased ApoB secretion is responsible for hepatic lipid accumulation [50, 51].

The results of lysosomal enzymes activity shown in Figure 5 indicate that EUCE increased the activity of lysosome enzymes by improving ER function. Through the protein degradation machinery activation, the requirement of protein folding can be relieved. GRP78, also known as binding immunoglobulin protein (Bip), is of a particular importance as a regulator of the ER stress response. GRP78 normally binds to three main transmembrane proteins: the protein kinase RNA- (PKR-) like ER protein kinase (PERK), activating transcription factor 6 (ATF-6), and inositol-requiring enzyme 1α (IRE-1α) [52]. In addition, GRP78 also serves as the master regulator of the ER stress response by binding and inactivating stress sensors at the luminal surface of the ER [53]. Initiation of ER stress response occurs when the quantity of misfolded or unfolded proteins in the ER exceeds the capacity of chaperone proteins that trigger the activation of UPR pathways. To reduce the accumulation of proteins in the ER, PERK phosphorylates eukaryotic initiation factor 2α (eIF-2α) to attenuate translation of proteins [54]. IRE-1α is related to genes involved in the transport of unfolded proteins out of the ER and in their degradation by ER-associated degradation (ERAD) pathway [55]. As reported, misfolded and unstable proteins in the ER are degraded by the ERAD pathway [56]. Lysosomes mediate degradation of the majority of intracellular proteins, and lysosomal activity is involved in the ERAD II pathway [57]. Through the stably maintained lysosomal activity, it may be suggested that EUCE regulates ER stress and its subsequent reduced bioactivation of CCl_4 to $CCl_3OO^{\bullet}$ by P450 2E1, which can activate ER stress in response.

In conclusion, the results of this study indicate that pretreatment with EUCE effectively decreases hepatic lipid accumulation induced by CCl_4. EUCE increases lysosomal enzyme activity, relieving the protein folding requirement leading to the attenuation of ER stress. The regulatory effect of ER stress is suggested to improve ApoB secretion as well as to regulate the biotransformation of CCl_4 and its resultant inhibition of ROS accumulation. Future research is necessary to unravel the mechanism of underlying the ability of EUCE to increase lysosomal enzyme activity, a suggested ER stress regulation mechanism.

Authors' Contribution

Chang-Feng Jin and Bo Li contributed equally to this work.

Conflict of Interests

The authors have no conflict of interests to disclose.

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Review Article

Increasing Whole Grain Intake as Part of Prevention and Treatment of Nonalcoholic Fatty Liver Disease

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In conjunction with the rise in rates of obesity, there has been an increase in the rate of nonalcoholic fatty liver disease (NAFLD). While NAFLD at least partially originates from poor diet, there is a lack of nutritional recommendations for patients with suspected or confirmed diagnosis of NAFLD, beyond eating a healthy diet, increasing physical activity, and emphasising weight loss. The limited current literature suggests that there may be opportunities to provide more tailored dietary advice for people diagnosed with or at risk of NAFLD. Epidemiological studies consistently find associations between whole grain intake and a reduced risk of obesity and related diseases, yet no work has been done on the potential of whole grains to prevent and/or be a part of the treatment for fatty liver diseases. In this review, we examine the potential and the current evidence for whole grains having an impact on NAFLD. Due to their nutrient and phytochemical composition, switching from consuming mainly refined grains to whole grains should be considered as part of the nutritional guidelines for patients diagnosed with or at risk for fatty liver disease.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the major liver diseases worldwide. Estimates of average prevalence vary from around 20–46% of the adult population in Westernised countries [1–3], with up to 45% in some ethnic groups [3, 4]. The prevalence of NAFLD increases up to 90% in obese populations, and given the worldwide rise in obesity, the incidence of NAFLD is likely to grow over the coming decades. While hepatic steatosis, an early stage of NAFLD, is often asymptomatic, in 20–30% of all cases, it can progress to nonalcoholic steatohepatitis (NASH). If untreated, NASH can progress to hepatic cirrhosis and reduced liver function and increased risk of early mortality [5]. Hepatic steatosis is characterised by a large number of fatty deposits in the liver. Critically, NAFLD in the hepatic steatosis phase can be reversed by lifestyle modification, while NASH is more difficult to treat though it can be reversed by bariatric surgery [6]. Thus, preventing the progression of hepatic steatosis to NASH is of primary importance.

Insulin resistance and oxidative stress are currently considered the primary mediators of NASH [7]. Other causes of NASH include total parenteral nutrition, certain drugs and industrial toxins, copper toxicity, and conditions characterized by extreme insulin resistance [8]. Peripheral insulin resistance leads to increased delivery of fatty acids to the liver, and high levels of circulating insulin interfere with the normal capacity of hepatic mitochondrial β-oxidation to metabolize fatty acids; these steps in turn lead to fat accumulation in the liver [9]. NASH most likely develops from a subsequent "second hit" whereby oxidative stress leads to lipid peroxidation in the liver [10]. Mitochondrial dysfunction and, more specifically, respiratory chain deficiency may also mediate the second hit by generating reactive oxygen species which oxidise fat deposits to release lipid peroxidation products toxic to hepatocytes and other hepatic cells [11]. The increase in oxidative stress and lipid peroxidation leads to greater inflammation, leading to activation of tumour necrosis factor-α and interleukin-6 mediated pathways and down regulation of hepatocyte autophagy (mitophagy, i.e.,

including the process of removing damaged mitochondria) [12].

While hepatic steatosis in itself may not lead to noticeable symptoms, the development of NASH is associated with significant morbidity and mortality and has limited therapeutic options [13–15]. The main causes of increased mortality among patients with NASH are cardiovascular disease, cancer, and liver failure [12]. Patients with NAFLD are at increased risk for other chronic diseases, including type 2 diabetes and cardiovascular disease [16, 17].

Key risk factors for NAFLD include (1) positive energy balance (excess energy intake and/or reduced physical activity), (2) obesity, (3) insulin resistance, and (4) hypertriglyceridemia. These factors are key targets for prevention and therapy of NAFLD though clinical treatment tends to focus on addressing the obesity or insulin resistance rather than NAFLD itself. As with all lifestyle-related diseases, improvement of diet should also play a role in its prevention and treatment.

The clinical diagnosis of NAFLD requires a liver biopsy; however, blood biomarkers (alanine aminotransferase and aspartate aminotransferase) [25] are commonly used in large-scale epidemiological studies that have examined the link between diet composition and NAFLD. While these measures have low specificity, they provide some indirect insight into the effects of diet on liver function. Presently, it is known that a high percentage of energy from fat, and especially saturated fat, may lead to liver damage and that high glycaemic foods and refined sugar and fructose may also lead to hepatic steatosis through increased de novo lipogenesis [26]. There is mixed evidence on the role of vitamins, with some suggestions that better vitamin D and E status is linked to lower incidence of NAFLD, but intervention studies that have examined the effects of vitamin E supplementation on NASH did not show conclusive improvement [7, 27, 28]. These studies have been supplemented with α-tocopherol in an attempt to treat NASH, which does not rule out a role for other E vitamers and prevention or treatment of NAFLD. Studies that have investigated the effects of dietary fat and carbohydrate have shown that isoenergetic diets with low-fat and low-carbohydrate diets decrease liver fat, while high-fat diets increase liver fat over two weeks [29, 30], and increased protein intake may also reduce intrahepatic lipids [31]. The mechanisms behind these effects are not well understood, and the long-term efficacy/safety of low-carbohydrate and high-protein diets has not been assessed although present clinical recommendations state that ketogenic diets should be avoided to prevent greater liver damage. A Mediterranean diet rich in vegetables, nonred meat protein sources, and polyunsaturated fat was found to improve insulin sensitivity and reduce liver fat without weight loss over 6 weeks [32], and a short-term exercise intervention reduced markers of NAFLD and apoptosis (alanine aminotransferase and cytokeratin 18), also without significant weight loss [33], suggesting that it may be possible to improve NAFLD without losing weight. Omega-3 fatty acid supplementation has also been used as a treatment for NAFLD, with some promising results [26], but it is outside the scope of this review.

Despite the strong recent efforts in the area of diet and NAFLD, present lifestyle recommendations for people diagnosed with NAFLD are generally limited to losing weight (e.g., through energy restriction) and increasing physical activity [34], as well as following national guidelines for a healthy diet [2, 35]. In many countries, this now includes the recommendation to eat at least half of all grain servings as whole grains, with gram recommendations ranging from at least 48 g/d to at least 75 g/2000 kcal [36].

While there are reviews that have covered the area of diet and physical activity for the treatment of NAFLD [26, 37, 38], none have examined whether increasing whole grains in the diet may be an effective strategy for prevention and treatment of NAFLD. This review aims to examine the evidence supporting the idea that a diet rich in whole grains is associated with a decrease in many of the risk factors and comorbidities associated with NAFLD and that replacing refined grains with whole grains in the diet could also be an important component of lifestyle changes that could successfully prevent and treat NAFLD.

2. Whole Grains

Whole grain cereals (whole grains; WG) are consistently associated with a decreased risk of NAFLD-related diseases, including obesity, diabetes, and cardiovascular disease [39]. The term "whole grains" covers the edible parts of the cereal grasses, including wheat, rice, corn, rye, barley, and oats, and also includes the pseudocereals (seeds that are used in a similar manner to cereal grains) (Table 1). The American Association of Cereal Chemists defines whole grains as cereals that "… shall consist of the intact, ground, cracked, or flaked caryopsis, whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportions as they exist in the intact caryopsis" [40]. Over the past 100–150 years, the most commonly consumed cereals are milled to remove the bran and the germ from the starch endosperm. This results in flour that has a longer shelf-life, has better organoleptic properties, and is easier to process than if all three components are present [41]. This also results in flour which is nutrient poor relative to the whole grain, especially in dietary fibre, vitamins, and minerals, as well as phytochemicals that may have health benefits (Table 1). Cereal grains represent one of the main staple foods worldwide, and improving the quality of cereal foods in the diet represents an excellent opportunity for improving health.

While several national dietary guidelines now include recommendations for increasing whole grain intake and replacing refined grains with whole grains [36], the average consumption remains relatively low at around one 16 g serving/d [42–44]. It should be noted that these data are from surveys taken before the 2005 Dietary Guidelines for Americans, which placed an increased emphasis on whole grains [39]. Thus, even though there is an increasing availability of a wide variety of whole grain foods and greater public awareness of the potential health benefits of whole

Table 1: Whole grains included under the American Association of Cereal Chemists definition, key macronutrients, and micronutrients that may play a role in nonalcoholic fatty liver disease. Refined wheat and rice are included as comparisons. Data are from the USDA database [18] unless otherwise stated. Note that these values are averages and do not represent the high varietal and seasonal variation that are normal for micronutrient contents of foods.

	Whole grains											Refined grains		
	Wheat	Rice	Corn	Rye	Oats	Barley	Sorghum	Millet	Quinoa[a]	Buckwheat[a]	Amaranth[a]	Wheat	Rice	Corn
Energy (kJ/100 g)	1418	1515	1515	1414	1628	1481	1418	1582	1540	1402	1552	1523	1498	1569
Carbohydrate (g/100 g)	72.6	76.2	76.9	75.9	66.3	73.5	74.6	72.9	64.2	70.6	65.3	76.3	79.2	82.8
Protein (g/100 g)	13.7	7.5	8.1	10.3	16.9	12.5	11.3	11	14.1	12.6	13.6	10.3	6.5	5.6
Fat (g/100 g)	1.9	2.7	3.6	1.6	6.9	2.3	3.3	4.2	6.1	3.1	7	1	0.5	1.4
Total dietary fibre (g/100 g)	12.2	3.4	7.3	15.1	10.6	17.3	6.3	8.5	7	10	6.7	3.1	1	1.9
Vitamin E (mg α-tocopherol/100 g)	0.8	0.6	0.4	0.9	0.7	0.6	0.1	0.1	2.4	0.3	1.2	0.1	0.1	0.2
Folate (μg/100 g)	44	20	25	38	56	19	—	85	184	54	82	10	6	48
Magnesium (mg/100 g)	138	143	127	110	177	133	—	114	197	251	248	22	35	18
Glycine betaine (mg/100 g)[b]	90	3	2	120	7	35	3	10	360	2	65	23	3	3
Free choline (mg/100 g)[b]	20	8	2	18	4	7	10	2	27	46	51	10	10	18

[a] Pseudocereals: botanically not true cereal grasses, but included in the whole grain definition due to their traditional use in the same way as cereals.

[b] Data from Bruce et al. [19] and unpublished results using the same liquid chromatography-tandem mass spectrometry method.

grains in the diet, recent data suggests that this has not been translated into greater whole grain consumption [45].

3. Whole Grains and Risk Factors for NAFLD

A considerable body of epidemiological work outlined below consistently reports that people who eat more whole grains have a reduced risk of cardiovascular disease, obesity, and diabetes, while some studies suggest that whole grains may also reduce markers of inflammation.

Several meta-analyses of epidemiological data have found that increasing whole grain intake reduces the risk of developing type 2 diabetes in the order of 16–26% compared to people eating the least amount of whole grains in their diet [20, 22, 46] (Table 2). Greater intake of whole grains is also associated with lower fasting C-peptide and insulin although not glycated haemoglobin (HbA1c) [47]. Recently, greater whole grain intake was associated with a 34% lower risk of deteriorating glucose tolerance [48].

A high whole grain intake is also linked to a decreased likelihood of being obese [49, 50]. There is evidence from both epidemiological [51] and randomised intervention trials [52, 53] that abdominal fat mass is reduced on a whole grain diet compared to refined grains, an effect which has not been reported for fruits and vegetables [51]. In a meta-analysis of three major US cohorts, a single serving (assumed to be 16 g) with an increase in whole grain intake resulted in a 0.25 kg decrease in body weight over four years, a small but nevertheless significant change [24]. Eating whole grains instead of refined grains while on a hypoenergetic diet does not increase weight loss although it does appear to improve fat loss [52, 54]. For NAFLD, reduction of body fat, rather than overall weight loss, may be more important, making these findings supporting the use of whole grains as a carbohydrate source during hypoenergetic diets highly interesting. Mechanisms behind whole grains leading to preferential loss of body fat remain to be determined.

There are some reports that whole grain intake correlates with reduced concentrations of some inflammation markers [55, 56] although this is not a universal finding [47]. Consuming >1 serving of whole grain was associated with reduced high-sensitivity C-reactive protein (hsCRP) in premenopausal women [56], while plasminogen activator inhibitor type 1 (PAI 1) and hsCRP were also decreased in adults who ate the most whole grains though fibrinogen was not associated with whole grain intake [55]. In women, whole grain intake was associated with reduced plasma CRP and tumour necrosis factor α-receptor 2; these effects were attenuated by waist circumference, insulin sensitivity, and 2 h-postload glucose [57], suggesting a strong link between inflammation and body composition/insulin resistance. Overall, epidemiological studies suggest that each 16 g serving of whole grain leads to a 7% decrease in CRP [58]. Evidence from intervention trials is less convincing, with one study finding reduced CRP in subjects with metabolic syndrome on a hypocaloric diet while eating whole grains compared to refined grains [52], and another finding IL-6 concentrations decreased, especially in overweight and female subjects as they had higher baseline IL-6 concentrations [59]. However, most other studies that have examined inflammation parameters have not found significant differences between refined grain versus whole grain consumption [60–62].

While epidemiological studies overwhelmingly report associations between highest intake of whole grains and reduced risk of cardiovascular diseases, diabetes, and obesity compared to those eating little or no whole grains [20, 21, 39], it should be acknowledged that intervention evidence for the effect of whole grains on markers of disease risk is mixed, with a number of studies finding a range of positive biomarker changes when eating whole grains compared to refined grains [52–54, 59, 62–66] while other studies have not found any benefits [60, 61, 67]. In some cases, this may be due to the difficulty of ensuring compliance in free-living settings [68], as well as the considerable heterogeneity of study design and populations used [20]. Meta-analyses of epidemiological and clinical trials point to an overall effect of diets rich in whole grains rather than refined grains reducing many of the risk factors for NAFLD (Table 2).

4. Potential Mechanisms of Action for Whole Grains in the Prevention and Treatment of NAFLD

Whole grains are higher in many nutrients and phytochemicals than their refined counterparts (Table 1), and several reviews have described possible mechanisms behind how they may be better for health [69, 70]. In the context of prevention or treatment of NAFLD, there are several possible mechanisms beyond better nutrient intake:

(i) reduction of energy intake (lower energy density compared to refined foods),

(ii) changes to and stimulation of gut microbiota, leading to increased production of short-chain fatty acids,

(iii) specific actions of phytochemicals (e.g., vitamins, phenolic acids, betaine),

(iv) synergistic interaction between different whole grain components (e.g., phenolic compounds interacting with stimulated gut microbiota).

In general, whole grain foods are less energy dense than their refined counterparts, though this depends on the amount of lipid from the germ; refined grains have the germ removed and a lower overall fat content. However, lipids from cereals are mainly unsaturated and so are not a primary cause for concern for the development of NAFLD. The slightly lower (around 5%) amount of carbohydrate in whole grains compared to refined grains may also reduce the amount of insulin required to handle the influx of glucose after a meal. The higher amount of fibre has also been suggested to increase intestinal bulking and thus influence satiety. There is little data to support an impact of whole grains on satiety [71], with the exception of rye [72–75]. However, whole grains are perceived as being more satiating by consumers [76], and this over the long term may reduce energy intake by reducing portion sizes.

Table 2: Associations between whole grain intake with risk factors for nonalcoholic fatty liver disease. Data are from meta-analyses only.

	Relative risk ratio/weighted mean difference compared to controls*	*P* value (after adjustment for potential confounders unless otherwise stated)	Median or average whole grain intake (high versus low; g whole grain/d)	Whole grains consumed	Study type	Number of cohorts/studies included in the meta-analysis	Reference
Cardiovascular disease (incidence)	0.79 (0.74, 0.85)	<0.001	44 versus 0	Mixed (mainly US studies)	Prospective cohort	9	[20]
	0.79 (0.73, 0.85)	<0.001	40 versus 3.2			7	[21]
Type 2 diabetes (incidence)	0.74 (0.69, 0.80)	<0.001	44 versus 0	Mixed (mainly US studies)	Prospective cohort	6	[20]
	0.79 (0.72, 0.87)	<0.001	32 versus 0+	Mixed (mainly US studies)	Prospective cohort	6	[22]
Fasting insulin (pmol/L)	−0.29 (−0.59, 0.01)	<0.001	>50 versus <20	Mixed	Intervention	10	[20]
	−0.011 (−0.015, −0.007)	<0.001	16 versus 0+	Mixed	Prospective cohort	14	[23]
Fasting glucose (mmol/L)	−0.93 (−1.65, −0.21)	<0.001	>50 versus <20	Mixed	Intervention	11	[20]
	−0.009 (−0.013, −0.005)	<0.001	16 versus 0+	Mixed	Prospective cohort	14	[23]
Total cholesterol (mmol/L)	−0.83 (−1.24, −0.42)	<0.001	>50 versus <20	Mixed	Intervention	16	[20]
LDL-cholesterol (mmol/L)	−0.72 (−1.34, −0.11)	<0.001	>50 versus <20	Mixed	Intervention	15	[20]
Weight gain (kg)	−0.18 (−0.54, 0.18)	ns	>50 versus <20	Mixed	Intervention	12	[20]
	−0.17 (−0.22, −0.11)	<0.001	16 versus 0+	Mixed (US cohorts)	Prospective cohort	3	[24]

*Highest versus lowest categories of whole grain intake in prospective cohort studies and weighted mean difference compared to controls in intervention studies.
**These values are not actual intake but are the difference intake estimated to lead to the corresponding change in biomarker concentration.

Several studies have now demonstrated that whole grains can change gut microbiota composition in humans, including *Bifidobacteria* and *Lactobacillus* [59, 66, 77, 78], with corresponding changes to gut microbiota metabolites including the short-chain fatty acid (SCFA) butyrate measured in faeces [79, 80] and plasma [81] and phenolic compounds measured in urine [79, 80]. Increased butyrate production is linked to improved tissue insulin sensitivity [82–84], and this may be one mechanism for how whole grains improve insulin sensitivity (see below). Increasing numbers of studies have found links between gut microbiota composition/metabolism and fatty liver disease [85–88], particularly for the role of lipopolysaccharide (LPS; a cell wall component of gram negative bacteria) and increased inflammation [89] and risk for NAFLD and NASH [90, 91]. Prevention of LPS uptake from the intestine may be limited by increased intestinal permeability; subjects with NAFLD or NASH are more likely to have increased intestinal permeability compared to healthy controls [92, 93]. LPS-induced inflammation via toll-like receptors has been linked to obesity and is proposed as a factor in the "second hit" of NAFLD leading to NASH [12]. Low-dose exposure to LPS may also increase hepatic triglyceride production and inhibit triglyceride export at higher doses [94] through inflammation-mediated interruption of fatty acid transporters [95]. Recently, it was proposed that increased circulating leptin leads to a hypersensitive response to circulating LPS [96], underlining the multitude of factors influencing the response to gut microbiota components. In a rat model of alcoholic fatty liver disease, rats fed with an oat-based diet had less liver fat accumulation than controls, and this effect was ascribed to thickening of the intestinal wall, thus preventing excessive ethanol transport across the intestine [97]. This mechanism may also inhibit transport of LPS across the intestinal barrier and prevent endotoxin-associated inflammation. Prebiotic fibre has been linked to changes in gut microbiota and NAFLD, and this may serve as another potential mechanism whereby whole grains protect against NAFLD. The addition of fructooligosaccharide (FOS) to the diet of mice with fatty liver induced by n-3 polyunsaturated fatty acid deficiency resulted in altered gut microbiota populations (including increased *Bifidobacteria*, as for some whole grain clinical studies [59, 77]) and reduced hepatic triglyceride accumulation. This effect was mediated via proliferator-activated receptor α-stimulation of fatty acid oxidation, possibly stimulated by the gut hormone GLP-1 [98]. The composition of gut microbiota and the integrity of the intestinal barrier may be key factors in the interaction between diet and NAFLD [89]. Diets rich in whole grains appear to consistently alter gut microbiota composition. Beyond the study of Keshavarzian et al. [97], no studies have examined whether whole grains can improve gut integrity.

A number of studies have demonstrated lower glycemic curves following a whole grain diet relative to refined wheat [81, 99], and the rate of glucose disappearance was increased in a morning glucose tolerance test after an evening meal of whole grain barley compared to white rice [100], possibly indicating improved insulin sensitivity. Beta-cell function was also found to be improved after a whole grain rye-based intervention [101]. These apparent improvements in glucose metabolism may be due to increased fermentation activity of the gut microbiota, through the action of butyrate, or due to micronutrients such as magnesium that are present in relatively high amounts in whole grain. The latter serves as cofactors for enzymes involved in glucose metabolism and insulin secretion [102]. It should also be noted that not all intervention studies have found improvements in markers of glucose metabolism (e.g., [60, 61]), and the outcomes may be highly dependent on the study population and design.

There are many phytochemicals that are abundant in whole grains but are only present in low amounts after refining. Of particular interest for potential treatment of NAFLD, whole grain wheat, rye, and quinoa have high amounts of the methyl donor glycine betaine (trimethylglycine, referred to henceforth by its common name "betaine") compared to other foods [19, 103]. High doses of oral betaine have been shown to reduce the severity of NAFLD in humans in case studies [104–106]. However, in one randomised controlled trial testing betaine supplementation there was no conclusive evidence for an effect on NASH or inflammation although betaine did reduce the steatosis grade [107]. The rationale for the effect of betaine is that it spares choline (which can be metabolised to betaine for remethylation of homocysteine) for synthesis into phosphatidylcholine, which in turn is a key component of VLDL particles for export of lipids from the liver. If too much choline is required for remethylation of homocysteine, then export of VLDL from the liver will be compromised, leading to a buildup of lipids in the liver. This type of mechanism has been demonstrated in rodent models of NAFLD where betaine supplementation reduces the severity of fatty liver in NAFLD models [108]. Higher plasma homocysteine itself has also been associated with steatosis and NASH [109, 110], and rodent studies suggest that Hyperhomocysteinaemia may cause liver damage and peroxidation [111]. However, elevated plasma homocysteine has not been associated with steatosis or NASH in all populations [112, 113], and genetics may play a role in determining whether hypohomocysteinaemia is a risk factor, especially for the progression to liver cirrhosis [114]. Whether betaine supplementation is effective in reducing steatosis or NASH in cases of hyperhomocysteinaemia is yet to be tested.

Wheat-based foods are the main source of betaine in the diet and account for 67% of total betaine intake in the New Zealand diet [103]. Choosing whole grains over refined grains could increase overall betaine intake by 1.5–3.3 fold. Although there are no existing recommendations for betaine intake, choline intake recommendations for adults in the US are 425–550 mg/d [115], recognizing that the pathway between phospholipid synthesis and remethylation of homocysteine is of importance for general health. It is uncertain what amount of dietary betaine is required to maintain optimal betaine status and this is likely to depend on choline and B-vitamin status as well. A whole grain rich diet (150 g/d) with 112 mg/d more betaine than the control refined grain diet increased plasma betaine [66] and a very high amount of wheat aleurone fraction (providing 279 mg/d more betaine than the control) reduced homocysteine and increased betaine, dimethylglycine, and methionine in healthy adults [116]. In

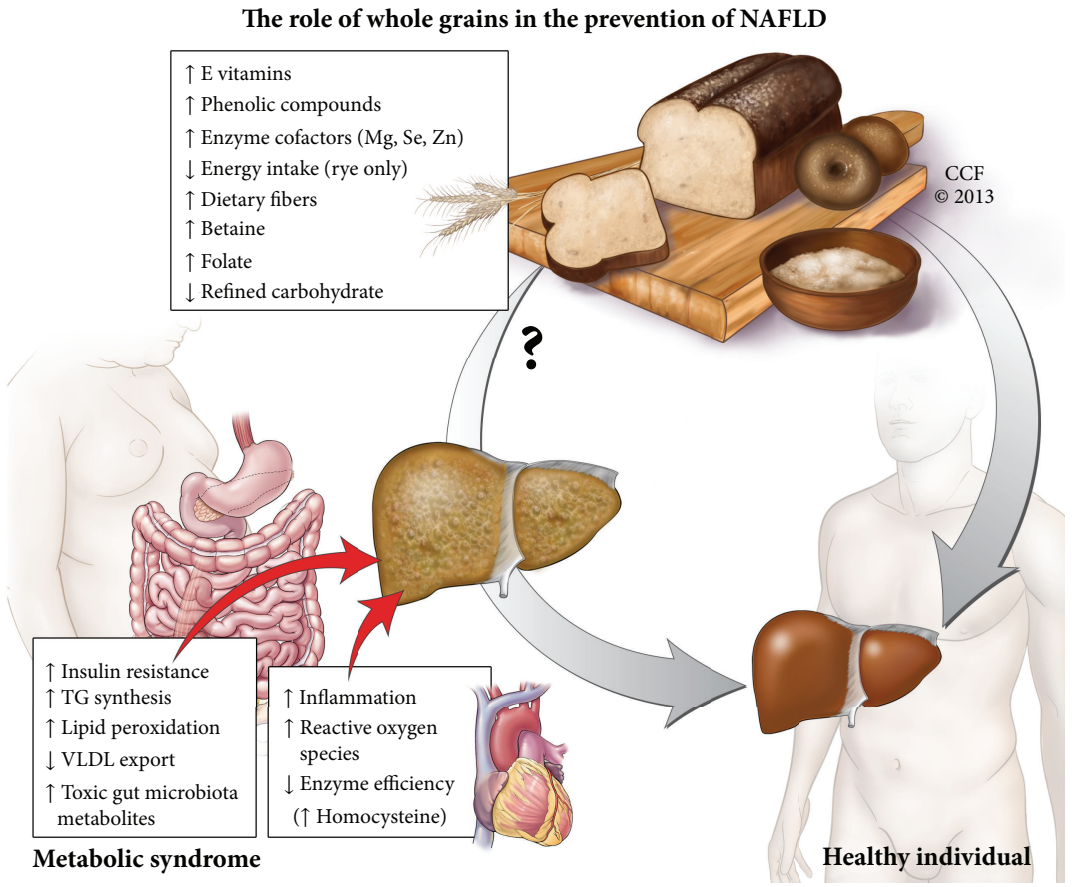

Figure 1: Whole grains may have an impact on nonalcoholic fatty liver disease through many complementary mechanisms. Choosing more whole grains over refined carbohydrate sources will increase the intake of many nutrients that are known to, or suggested to, play a role in preventing fatty liver diseases and related comorbidities. While yet to be studied directly, it is probable that a diet rich in whole grains would play a role in the prevention of fatty liver diseases. Whether they could be a biologically active part of a diet to treat nonalcoholic fatty liver disease remains to be investigated.

epidemiology, greater whole grain intake has also been associated with decreased homocysteine [47]. Low plasma betaine and high plasma choline were associated with increased BMI, body fat, and serum triglyceride, while increased plasma betaine was associated with increased plasma folate, serum HDL, and decreased plasma homocysteine [117]. The one-carbon metabolic pathway may also interact with the gut microbiota, which may also play an important role in the development of fatty liver in humans on a choline deficient diet [118]. Given that betaine reverses fatty liver in rodents on a choline-deficient diet, whole grains as a main source of dietary betaine and an ingredient that can alter gut microbiota, mean that several different but interlinked mechanisms for preventing fatty liver may potentially occur (Figure 1). Independent of whole grain intake, coeliac disease may be a risk factor for NAFLD, and elevated concentrations of transaminases are common in people with coeliac disease [119] though this could also be due to intestinal damage. The gluten-induced gastrointestinal inflammation is hypothesised to lead to increased gut leakage [91] and increased blood LPS, and people diagnosed with NAFLD and coeliac disease often improve after they are placed on a gluten-free diet. Monitoring coeliac disease is suggested for NAFLD patients, even when no other metabolic risk factor is present [119]. This being the case, it should be noted that cereals that form the basis of gluten-free diets (i.e., avoidance of wheat, rye, barley and oats) are generally very low in betaine ([19] and Table 1), with the exception of the South American pseudocereals amaranth and quinoa. In the case of individuals with coeliac disease or gluten intolerance who are also at risk of NAFLD, regular consumption of these two pseudocereals may be advisable.

Recently, Fardet proposed the concept of lipotropes, compounds that may play a role in preventing excessive hepatic fat accumulation [120], including betaine, choline, myo-inositol, methionine, magnesium, niacin, pantothenic acid, folate, and total polyphenol content. Cereal-based foods, and especially whole grains, are considered the most economic source of lipotropic compounds [121]. However, this hypothesis is currently based on animal data and it remains to be seen if the lipotropic index translates it to the clinical setting. Nevertheless it is clear that whole grains are always higher

in these "lipotropic" compounds compared to their refined equivalents, and when cereals make up a large part of our energy intake, this may have consequences for NAFLD.

Other compounds present in high amounts in cereals may play a role in preventing NAFLD. In particular, whole grains are rich in phenolic compounds, mainly ferulic acid. *In vitro* and animal studies suggest that ferulic acid conjugates can reduce inflammation via inhibition of the transcription factor NF-κB and activation of proinflammatory pathways [122]. However, ferulic acid itself had little effect in a Caco-2 cell model of intestinal inflammation though flavonoids did [123]. A fermented whole grain wheat bread was found to reduce *ex-vivo* cytokine production in blood, and this was associated with greater ferulic acid bioavailability [124]. In cereals, the phenolic acids are mainly (>95%) bound to fibre and are not thought to be released from the matrix during intestinal transit, thus leading to a low bioavailability of <5% [125]. It is possible that even if phenolic acids are not absorbed, they do play a role in modulating gut microbiota and improving the chemistry of the intestinal milieu [126]. While ferulic acid is a strong antioxidant, it is unlikely that the amount absorbed has a meaningful effect on overall antioxidant status through classical mechanisms [127, 128]. Another group of phenolic compounds, the alkylresorcinols (phenolic lipids), is present in high amounts in wheat and rye and has around 60% absorption by humans [129]. There is relatively little information about any bioactivity *in vivo* [130] although one study where rats were fed rye alkylresorcinols found that high doses (0.4% of total diet) led to a dramatic decrease in total liver lipids and an increase in liver γ-tocopherol via competitive inhibition of CYP450-mediated β-oxidation [131]. In adipocyte models, alkylresorcinols were also found to inhibit triglyceride synthesis [132] and inhibit hormone sensitive lipase-mediated-lipolysis of triglycerides [133], suggesting that they may both prevent excessive triglyceride accumulation and prevent elevated circulating concentrations of nonesterified fatty acids (NEFA) [134]. Alkylresorcinol intake was associated with decreased plasma NEFA after a whole grain intervention [68] though more *in vitro* and *in vivo* studies are needed to confirm this potential mechanism. Oats contain phenolic analogues of the anti-inflammatory drug tranilast, avenanthramides. Avenanthramides also have anti-inflammatory properties *in vivo* [135], though concentrations in oats are low, and a high intake of oats would likely be required to observe an acute effect. Avenanthramides may in part be responsible for the effect of oats in protecting against ethanol damage to the liver of rodents, otherwise ascribed to an improvement of the intestinal barrier [97].

It is also likely that the different components of whole grains act together synergistically. A whole grain wheat diet increased faecal Bifidobacteria populations in humans compared to wheat bran [77], and whole grain wheat decreased cholesterol in a rat model, whereas wheat bran did not [136]. It is also speculated that phenolic compounds and dietary fibre from whole grains differentially interact with the host microbiota to improve overall host-microbiota interactions [126]. The synergy between the different components of the whole grain may account for why they are associated with reduced risk of a wide range of diseases.

Whole grain foods potentially contain a variety of anti-nutrients and toxins, as the outer protective layer of the grain is included, and this is exposed to soil, fungal infections, and pesticides. In practice, cereal raw materials are strictly monitored to ensure that known mycotoxins and pesticides are below local thresholds of concern [137, 138], and millers often remove the outer 1-2% of the grain before making "whole grain" flour to substantially reduce the likelihood of contaminants being present [139]. Whole grains are also high in phytate, which decreases mineral bioavailability, both micronutrient minerals and heavy metals [140], and rice bran phytate has been demonstrated to reduce lead poisoning in a rat model [141]. While contaminated grains could be a source of toxins that could cause liver damage, there is no population-based evidence to suggest that current thresholds for monitoring contaminants are inadequate.

In the diet and NAFLD literature, there is some concern that diets higher in carbohydrates may be linked to the increased incidence of NAFLD [142, 143] and that low-carbohydrate diets may lead to greater fat loss and improvement in insulin sensitivity [29, 144]. However, these studies do not describe the type of carbohydrate in the diet, nor the origins. While sucrose, refined white wheat flour, whole grain flour, and potatoes may all be classed as "carbohydrate rich", they all lead to different metabolic responses, even before accounting for different food processing methods. We therefore suggest that the source of carbohydrates may be critical and that the greater nutrient content of whole grains makes them a "positive" source of energy for people with NAFLD compared to other carbohydrate rich foods that are dietary staples in the Western diet and may exacerbate fatty liver disease.

5. Conclusions

Presently the only empirically based dietary advice for patients with NAFLD is to eat a hypoenergetic diet and lose weight, and there is little evidence for what sort of foods should be used to achieve this with the best outcome. People who consume diets rich in whole grains tend to have a lower risk of many of the comorbidities associated with NAFLD, including NASH, and all whole grains contain higher amounts of compounds which may help reduce liver fat and protect against the inflammation that is thought to act as the second hit that leads to the progression from steatosis to NASH. Since there is no direct evidence that a whole grain diet may help to protect or treat NAFLD, clinical studies comparing whole grains with refined grains for preventing and treating NAFLD are needed. Given the multiple potential mechanisms where whole grains may prevent or treat risk factors associated with NAFLD (Figure 1), there is a good basis to recommend that people with or at risk of NAFLD should choose whole grains over refined grains in their diet.

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Clinical Study

Hepatic Steatosis, Carbohydrate Intake, and Food Quotient in Patients with NAFLD

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Is steatosis related to the spontaneous carbohydrate intake in patients with NAFLD? We performed dietary records for 24 patients with NAFLD, 3 months after their liver biopsy was performed and before the deliverance of a dietary advice. The food quotient, indicator of the proportion of calories from carbohydrates, was calculated as (1.00×% calories from carbohydrates/100) + (0.70×% calories from lipids/100) + (0.81×% calories from proteins/100). The associations between diet variables and steatosis% on the hepatic biopsies were tested by regression analysis, and diet variables were compared according to the presence of fibrosis. The subjects displayed a large range of steatosis, 50.5% ± 25.5 [10–90], correlated with their energy intake (1993 ± 597 kcal/d, $r = 0.41$, $P < 0.05$) and food quotient (0.85 ± 0.02, $r = 0.42$, $P < 0.05$), which remained significant with both variables by a multivariate regression analysis ($r = 0.51$, $P < 0.05$). For the 17/24 patients with a hepatic fibrosis, the energy intake was lower (fibrosis: 1863 ± 503 versus others: 2382 ± 733 kcal/d, $P < 0.05$), and their food quotients did not differ from patients without fibrosis. Hepatic steatosis was related to the energy and carbohydrate intakes in our patients; the role of dietary carbohydrates was detectable in the range of usual carbohydrate intake: 32% to 58% calories.

1. Introduction

The high prevalence of nonalcoholic fatty liver diseases (NAFLD) is now well recognized, involving 15% (ultrasound study in China [1]) to 34% (MR spectroscopy in the USA [2]) of adults. Not only serious late consequences, as liver fibrosis [3] and hepatocellular carcinoma [4], but also high rates of cardiovascular events [5], make NAFLD a relevant public health issue. Lifestyle modifications, predominantly dietary, are considered as the first line of the therapy [6]; however, beside the importance of losing excessive weight, the dietary counselling for NAFLD is not consensual, as reflected by several recent reviews [7–9]. The reduction of liver fat is a logical objective, 26% of which arise from De Novo Lipogenesis and 15% from the diet, so less dietary carbohydrates and/or lipids may help [10]; however, their proportion is debatable.

The Nutrition and Hepatology Teams of the Centre Hospitalier Universitaire de Bordeaux cooperatively follow patients with NAFLD: once the diagnosis of NAFLD is stated by the hepatologist, the patients are referred to the Nutrition Team; they are interviewed by a dietician before the delivery of dietary advice. This gave us the opportunity to test whether the degree of steatosis and the presence of hepatic fibrosis could be related to their spontaneous energy intake and to the proportion of energy from carbohydrate, as reflected by the food quotient.

2. Materials and Methods

The dietary interviews were performed by dieticians, who performed a seven-day recall of dietary intakes, using a BillNutIV software for the analysis of the nutrient intakes, in 24 patients with NAFLD, 3 ± 2 months after their hepatic

biopsy was performed, and before the deliverance of a dietary advice. Other causes of liver diseases (virus and drugs) were excluded, as were the subjects who declared an excessive alcohol consumption (>20 g/d for women and 30 g/d for men) and the subjects who had known diabetes that might have led them to restrain their carbohydrate intake.

The food quotient, indicator of the proportion of calories from carbohydrates, was calculated as (1.00×% calories from carbohydrates/100) + (0.70×% calories from lipids/100) + (0.81×% calories from proteins/100) [11].

Steatosis was evaluated by a senior pathologist as a percent of hepatocytes containing fat droplets, either macrovacuolar or microvascular, on liver sections stained with HES (hematin-eosin-saffron) of transparietal liver biopsies. The presence or absence of fibrosis (any stage, including cirrhosis) was evaluated histologically by the same pathologist, using Masson's trichromic stain.

The associations between diet variables and steatosis% on the hepatic biopsies were tested by regression analysis, and diet variables were compared according to the presence of fibrosis on the biopsies.

3. Results and Discussion

Twenty-four subjects (15 men, 9 women, age 45 ± 13 yrs) were referred to the Nutrition Team after the diagnosis of NAFLD was stated on a liver biopsy. They were overweight (BMI: 29.7±3.8; waist circumference: men 100±7 cm; women 100 ± 8 cm), with abnormal liver tests (ALAT: 72 ± 53 IU/L, GGT: 117±98 IU/L). According to the exclusion criteria, they were not known as diabetic (fasting glycemia: 5.4 ± 0.8 mM), and their alcohol intake was below the recommended limits in France: mean 2 ± 5 g/day.

The steatosis% was 50.5 ± 25.5 [10–90]. The relations between the steatosis% on the hepatic biopsy and the dietary calorie intake (kcal/day) and the food quotient were both significant ($r = 0.41$ and 0.42, resp., both $P < 0.05$) as depicted in Figures 1(a) and 1(b) ($r = 0.51$, $P = 0.039$ with both by multivariate regression analysis). This confirms that less calories *and less carbohydrates* in the diet are associated with less fat in the liver of the patients with NAFLD. The steatosis% was also correlated to the carbohydrate intakes expressed as grams/day ($r = 0.49$, $P = 0.015$, Figure 1(c)), whereas the correlations with the intakes of lipids ($r = 0.15$), proteins ($r = 0.30$) and simple sugars ($r = 0.10$) were all far from significance. De novo lipogenesis is considered as a small contributor to the accumulation of fat in the whole body [12], but several investigators have reported that it may be an important contributor for liver fat [13–15], and two recent reports using proton magnetic resonance spectroscopy emphasized this importance. Browning et al. demonstrated a greater reduction of hepatic triglycerides after 2 weeks on a low-carbohydrate diet (26 g carbohydrates/day) than during a 50% carbohydrate low-calorie diet in 18 patients with NAFLD [16]. On the other hand, Sevastianova et al. have shown that only three weeks of carbohydrates overfeeding could increase liver fat by +27% contrasted with a +2% body weight gain [17]. In our patients, the relation between liver fat and dietary carbohydrates applied to a more usual range of carbohydrate proportion: our extreme food quotients were 0.82 (33% carb, 44% lipids, 23% protein, and 20% liver steatosis) and 0.89 (58% carb, 30% lipids, 12% protein, and 80% liver steatosis), which suggests that even moderate reductions of dietary carbohydrate may help to reduce liver fat in NAFLD. However, hepatic steatosis is, by itself, a benign condition [18], and the real offends are hepatic inflammation and fibrosis that may not rely on the same mechanisms.

Table 1: Comparison of the subjects with versus without liver fibrosis.

	With fibrosis	No fibrosis	P
n	17	7	
Men	10	5	NS
Age (yrs)	49 ± 11	34 ± 12	<0.05
ALAT (IU/L)	50 ± 23	127 ± 69	<0.001
GGT (IU/L)	125 ± 98	95 ± 102	NS
Fasting glycaemia (mmol/L)	5.5 ± 0.7	5.1 ± 0.4	NS
Steatosis% on the hepatic biopsy	45 ± 25	63 ± 21	NS
Body Mass Index (kg/m^2)	30.6 ± 3.4	27.4 ± 4.0	NS (0.06)
Maximal BMI during life	33.2 ± 3.4	28.7 ± 4.0	<0.01
Waist circumference (cm)	101 ± 8	96 ± 6	NS
Energy intake (kcal/day)	1863 ± 503	2382 ± 73	<0.05
Carbohydrate intake (grams/day)	204 ± 63	290 ± 117	<0.05
Simple sugars (grams/day)	70 ± 40	103 ± 60	NS
Lipid intakes (grams/day)	173 ± 55	194 ± 35	NS
Protein intakes (grams/day)	88 ± 20	110 ± 32	NS (0.06)
Food quotient	0.85 ± 0.02	0.86 ± 0.01	NS

Seventeen of the 24 patients had liver fibrosis, their characteristics are compared to those of nonfibrotic patients in Table 1. In our patients, fibrosis was not related to high calorie intakes, that were even lower (1860 ± 471 kcal/day versus 2380 ± 690 if no fibrosis, $P < 0.05$), or to food quotients, that did not differ (fibrosis: 0.85 ± 0.02, no fibrosis: 0.86 ± 0.02, NS). The older age of the patients with fibrosis may have contributed to both their lower calorie intakes and to their hepatic fibrosis as their exposure to steatosis had a presumably longer duration. These negative results do not question the interest of a well-balanced weight-losing lifestyle intervention, which was proven to improve steatosis and inflammation by a randomized controlled trial [19]. Because our effective was limited, we cannot rule out that very high intakes [20] or some specific forms of carbohydrates like fructose [21] may favor hepatic fibrosis. But by contrasting this with the relation to the steatosis%, we failed to detect a change in the lipid/glucide proportion in our patients with fibrosis; their absolute carbohydrate intakes were even significantly lower as shown in Table 1.

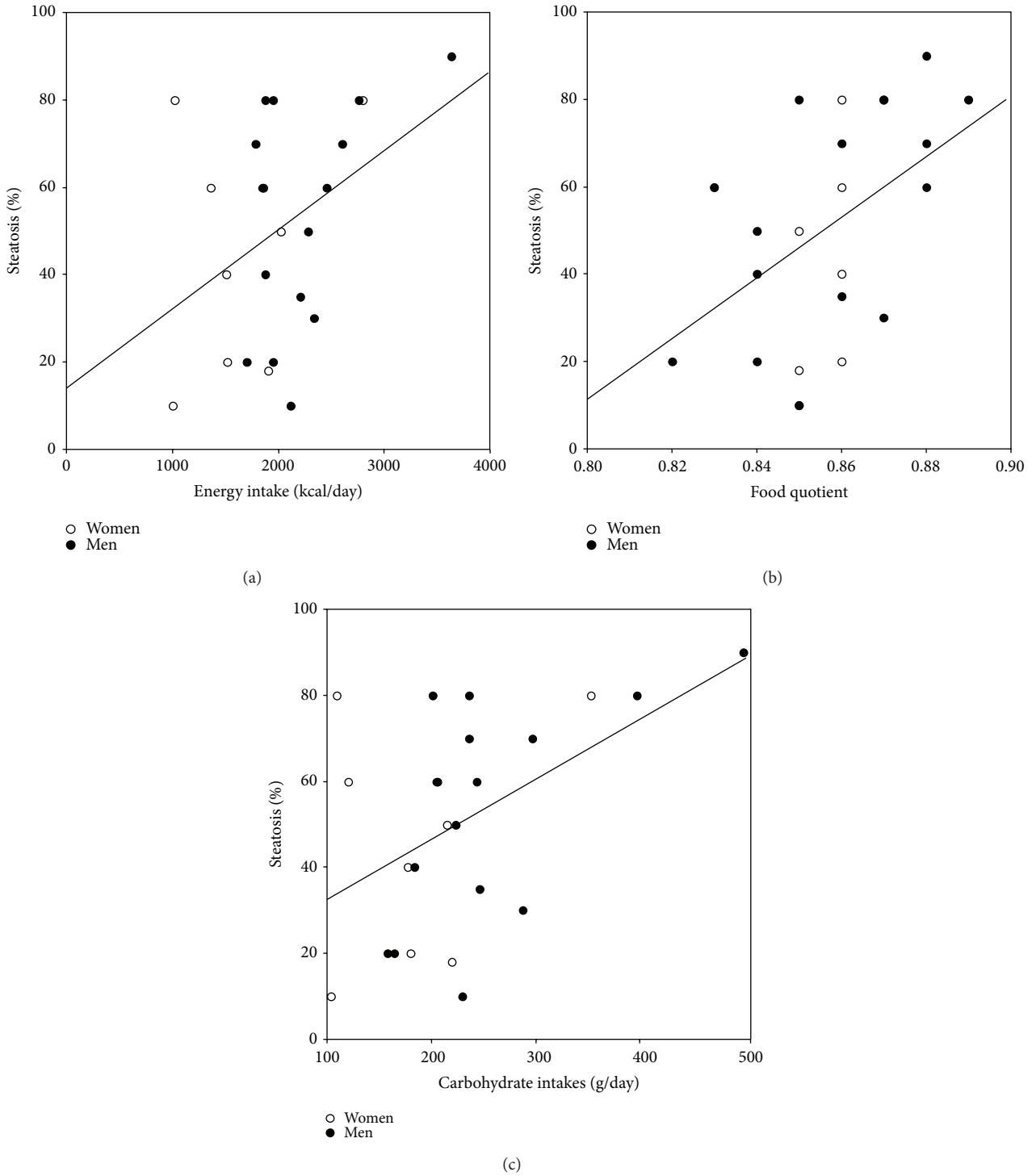

Figure 1: Steatosis% (y-axis) as a function of the energy intakes (x-axis, (a)), food quotients (x-axis, (b)), and carbohydrate intakes (x-axis, (c)) in 24 patients with NAFLD (women—open circles, men—closed circles).

On the long term, very low-carbohydrate diets are limited by high attrition rates [22] and a questionable risk of coronary heart disease [23]. We therefore feel that reducing carbohydrate is an interesting track in the field of NAFLD, but it needs caution for a generalized long-term application.

4. Conclusions

Hepatic steatosis was related to the energy and carbohydrate intakes in our patients. The role of dietary carbohydrates was detectable in the range of usual intake: 32% to 58% calories from carbohydrates, but the energy intakes and the

food quotients were not higher in the patients with hepatic fibrosis, the real offender. More information seems required before considering that reducing carbohydrates from the diet is beneficial on the long term in patients with NAFLD.

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Research Article

Intrahepatic Lipid Content and Insulin Resistance Are More Strongly Associated with Impaired NEFA Suppression after Oral Glucose Loading Than with Fasting NEFA Levels in Healthy Older Individuals

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Introduction. The mechanisms underlying the association between insulin resistance and intrahepatic lipid (IHL) accumulation are not completely understood. We sought to determine whether this association was explained by differences in fasting nonesterified fatty acid (NEFA) levels and/or NEFA suppression after oral glucose loading. *Materials and Methods*. We performed a cross-sectional analysis of 70 healthy participants in the Hertfordshire Physical Activity Trial (39 males, age 71.3 ± 2.4 years) who underwent oral glucose tolerance testing with glucose, insulin, and NEFA levels measured over two hours. IHL was quantified with magnetic resonance spectroscopy. Insulin sensitivity was measured with the oral glucose insulin sensitivity (OGIS) model, the leptin: adiponectin ratio (LAR), and the homeostasis model assessment (HOMA). *Results*. Measures of insulin sensitivity were not associated with fasting NEFA levels, but OGIS was strongly associated with NEFA suppression at 30 minutes and strongly inversely associated with IHL. Moreover, LAR was strongly inversely associated with NEFA suppression and strongly associated with IHL. This latter association (beta = 1.11 [1.01, 1.21], $P = 0.026$) was explained by reduced NEFA suppression ($P = 0.24$ after adjustment). *Conclusions*. Impaired postprandial NEFA suppression, but not fasting NEFA, contributes to the strong and well-established association between whole body insulin resistance and liver fat accumulation.

1. Introduction

Excess intrahepatic lipid (IHL) accumulation (nonalcoholic fatty liver disease, NAFLD) is an important component of the spectrum of metabolic derangements associated with central obesity and insulin resistance [1]. Studies elucidating the mechanistic basis for these associations have suggested that increased circulating nonesterified fatty acids (NEFAs) lead to elevations in IHL [2] and that these NEFAs are generated predominantly from lipolysis in white adipose tissue [3]. Inhibition of glucose oxidation by fatty acids is known to be an important feature of the glucose-fatty acid (or Randle) cycle [4], such that increases in plasma NEFA levels during starvation or in the diabetic state result in greater fat oxidation at the expense of glucose oxidation. Although insulin-mediated suppression of circulating NEFA levels is a

robust marker of adipocyte insulin sensitivity [5, 6], elevated fasting NEFA levels are generally considered to be the cause of hepatic fat accumulation in obese insulin-resistant states [7]. Furthermore, a recent study of 42 nonobese adults which measured postabsorptive fatty acid disposal with labeled palmitate found no association between this and whole body or peripheral insulin sensitivity [8]. A formal comparison of the strengths of the associations between fasting and suppressed NEFA levels is therefore warranted. Our first objective was to compare the strengths of the associations, if any, between whole body insulin sensitivity (as the exposure) and fasting NEFA levels versus NEFA suppression after oral glucose loading (at 30 and 60 minutes, as the "outcomes"). For this, we conducted a post hoc, cross-sectional analysis of metabolic and anthropometric data from a cohort of healthy older adults who participated in the Hertfordshire Physical Activity Trial (HPAT) [9].

NEFA suppression is impaired in type 2 diabetes [10] and NAFLD [11], but whether the well-established associations between insulin resistance, impaired NEFA suppression, and IHL persist in apparently healthy older individuals without prevalent NAFLD or diabetes is not known. Also, the extent to which impaired NEFA suppression modulates the accumulation of IHL has not previously been determined. Chronic inflammation within adipose tissue has been implicated in the pathogenesis of peripheral insulin resistance [12], and levels of fat-derived hormones (specifically the leptin : adiponectin ratio, LAR) have recently been shown to correlate well with clamp measures of whole body insulin sensitivity [13]. Therefore, our second objective was to estimate the associations between model-based measures of insulin resistance and impaired NEFA suppression as exposures and IHL as the outcome and to determine the extent to which the associations between insulin resistance and IHL were explained by variations in NEFA suppression.

2. Methods

The rationale and design for the Hertfordshire Physical Activity Trial (ISRCTN 60986572) have been described previously [9]. Data reported here relate to post hoc, cross-sectional analyses of volunteers' anthropometric and metabolic characteristics at the time of their entry into the study. Each participant provided written informed consent. The original study protocol was approved by the Hertfordshire Research Ethics Committee (LREC ref. 05/Q0201/23).

Trial participants were recruited from the Hertfordshire Cohort Study, consisting of men and women born in Hertfordshire, UK, between 1931 and 39 and still residing there [14]. Specifically, those who were deemed to be potentially suitable by their general practitioner for inclusion in a supervised aerobic exercise programme and who lived within ten miles of the exercise facility were invited to participate, as described previously [9]. Those with known diabetes, untreated or unstable ischaemic heart disease, or any medical condition that would preclude participation in an exercise programme were excluded from the trial. However, participants with incident diabetes (diagnosed at the time of entry to this study) were included in these analyses. Recruits attended the clinical research facility after an overnight fast. Of 106 who attended the screening visit, six were deemed to be unsuitable for the study because of poor mobility, preexisting diabetes, symptoms or signs suggestive of untreated ischaemic heart disease, or a combination of these factors were excluded. Of the remaining 100, MR imaging and spectroscopy were not performed on 30 individuals who had claustrophobia, cardiac pacemakers, or metal implants. Thus, 70 participants who enrolled in the study had baseline liver spectroscopy measures performed and constitute the cohort described herein.

All measurements were undertaken by trained staff adhering to standard operating procedures. Weight was measured on a Tanita (Tokyo, Japan) scale and height with a Seca (Hamburg, Germany) wall-mounted stadiometer. Waist circumference was measured using a D-loop nonstretch fibreglass tape measure and defined as the midpoint between the lower costal margin and the level of the superior iliac crests. Blood pressure was measured with an oscillometric device (Omron, Kyoto, Japan) using the right arm, after participants were seated quietly for five minutes. A dual energy X-ray absorptiometry (DEXA) scan (Lunar Prodigy Advance, GE Healthcare, Bedford, UK) was used to measure lean mass and body fat percentage [15]. Magnetic resonance measures of intrahepatic lipid (IHL) and visceral adipose tissue (VAT) were conducted on a whole body Siemens 3T Tim Trio scanner (Erlangen, Germany), as described previously [9]. A questionnaire was used to quantify alcohol consumption in units per week.

A standard 75 g oral glucose tolerance test (OGTT) was performed. Fasting samples were taken for glucose, insulin, C-peptide, lipid profile, NEFA, leptin, and adiponectin. Glucose was measured using a hexokinase assay (Siemens, Frimley, UK). Insulin and C-peptide were measured using a fluorometric autoDELFIA immunoassay (PerkinElmer Life Sciences, Turku, Finland). NEFA levels were measured on a colorimetric assay (Roche Diagnostics, Burgess Hill, UK). Leptin and adiponectin were both measured using a DELFIA assay (R&D Systems Europe, Abingdon, UK). After ingestion of glucose, further samples were taken every 30 minutes over two hours. Samples for glucose and lipid profiles were processed immediately, while those for insulin, C-peptide, NEFA, leptin, and adiponectin were spun and frozen for subsequent batch analysis. All samples were processed in the same laboratory. The oral glucose insulin sensitivity (OGIS) model [16] was used to determine peripheral insulin sensitivity based on dynamic insulin and glucose responses during the OGTT, primarily mediated through insulin effects on muscle. Additionally, we used the leptin : adiponectin ratio [13] as an index of whole body insulin sensitivity and the homeostasis model assessment (HOMA) as an index of hepatic insulin sensitivity [17]. The degree of insulin-mediated NEFA suppression was determined by calculating the percentage reduction in NEFA levels 30 and 60 minutes after glucose loading. The area under the concentration curve (AUC) for NEFA during the OGTT was calculated with the trapezium rule.

The anthropometric and metabolic characteristics of the study participants were summarised using means and standard deviations. To estimate the association between each of these characteristics and IHL, linear regression was used with log(IHL) as the outcome, and each characteristic standardised to have mean 0 and variance 1. The models also included age, gender, alcohol consumption (units per week, self-reported), and, where relevant, MRI-derived visceral fat area. For each exposure, the beta coefficient and 95% confidence limits were exponentiated, giving a ratio of geometric mean IHL per standard deviation increase in the exposure. The associations between standardised measures of insulin sensitivity (OGIS, LAR, and HOMA) and IHL were estimated using the same method, with adjustment for age, gender, MRI-derived visceral fat area, and alcohol consumption. The potential confounding effect of NEFA suppression at 30 and 60 minutes was also explored.

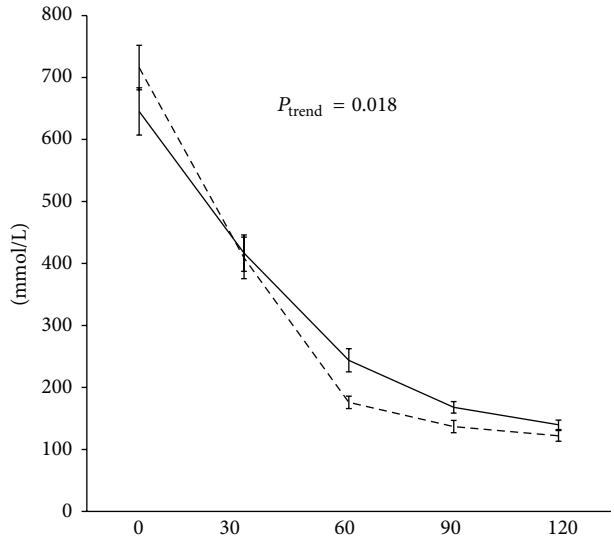

Figure 1: NEFA levels during standard 120-minute 75 g oral glucose tolerance test in participants with intrahepatic lipid ≤5.5% (dashed line) versus those with intrahepatic lipid >5.5%, that is, NAFLD (solid line). Data are presented as mean ± SE. P_{trend} values are derived from linear regression modelling with IHL treated as a continuous outcome variable and the exposure being the area under the NEFA curve, adjusted for age, gender, alcohol consumption, and visceral fat area.

3. Results

Of the 70 HPAT participants included in these analyses, 39 were men. Mean ± SD age was 71.3 ± 2.4 years. Systolic and diastolic blood pressures were 137 ± 18 and 75 ± 9 mmHg, respectively. The median IHL content was 3.6% (range 0.2–34.5%), while 42% of participants had IHL >5.5%, thus exceeding the arbitrary diagnostic threshold for NAFLD [18]. Three participants were found to have incident, asymptomatic type 2 diabetes based on OGTT results and were included in all analyses. Data relating to other anthropometric and metabolic characteristics of the cohort are summarised in Table 1. The associations between these characteristics and IHL are also shown in Table 1. These associations have been standardised in order to allow a comparison of their relative strengths. So, for example, each standard deviation rise in LAR was associated with a 49% increase in IHL, while each standard deviation rise in adiponectin was associated with a 48% reduction in IHL. Different measures of adiposity were positively associated with increased IHL, as expected. There were significant associations between each measure of insulin sensitivity and IHL, in the anticipated directions. NEFA suppression after oral glucose loading was inversely associated with IHL as shown in Table 1 and Figure 1. Results were similar for unadjusted analyses (data not shown).

To assess the relative strengths of the associations between insulin sensitivity and fasting as opposed to suppressed NEFA levels, we standardised OGIS, LAR, and HOMA. There were no significant associations between any indices of insulin sensitivity and fasting NEFA levels (Table 2), nor did HOMA correlate with measures of NEFA suppression. The associations between both OGIS and LAR and NEFA suppression were stronger for the 30-minute than the 60-minute values (Table 2) and were not significant for any of the 120-minute values (data not shown). OGIS and LAR had equivalent (though opposing) strengths of association with NEFA suppression at 30 minutes although the significance of the inverse associations at 60 minutes for LAR was strengthened after adjusting for age, gender, MR VAT, and alcohol consumption.

In order to determine the extent to which impaired NEFA suppression contributes to the observed inverse associations between insulin sensitivity (as exposure) and liver fat (as outcome), we compared these associations before and after adjusting for NEFA suppression (Table 3). After adjusting for NEFA suppression at 30 minutes, the positive association between LAR and IHL was attenuated and lost statistical significance. However, when OGIS or HOMA was treated as the insulin sensitivity measure, there was no attenuation of the association with IHL after adjusting for NEFA suppression. These results were similar after adjusting for NEFA suppression at 60 minutes, as shown. In order to avoid any confounding effects of medications known to influence lipid metabolism, we conducted subgroup analyses excluding those taking statin medications (n = 14, 20%) and those taking beta-blockers (n = 12, 17%), but these did not change our findings (data not shown).

4. Discussion

We found strong and consistent associations between OGIS and LAR (but not HOMA) and NEFA suppression. The absence of any associations with fasting NEFA levels is consistent with other recent observations [19] and suggests that fasting NEFA levels may be influenced by other factors such as catecholamine or growth hormone levels, whereas postprandial NEFA suppression is predominantly determined by insulin. NEFA levels are very promptly suppressed by insulin,

Table 1: Anthropometric and metabolic characteristics of study participants and their associations (after standardization) with intrahepatic lipid as the outcome measure.

Variable	Mean ± SD	Range	Standardised beta[a]	95% C.I.	*P*
Weight (Kg)[b]	76.0 ± 13.8	48.6–111.7	2.51	[1.86, 3.38]	<0.001
BMI (kg m^{-2})[b]	26.6 ± 3.5	20.7–37.8	2.03	[1.56, 2.64]	<0.001
Waist (cm)[b]	96.7 ± 11.9	72.3–120.5	2.70	[2.05, 3.55]	<0.001
Total fat (%)[b]	32.8 ± 7.5	15.9–48.2	2.26	[1.59, 3.22]	<0.001
MRI VAT (cm^2)[b]	127.7 ± 65.9	29.2–285.3	2.64	[2.02, 3.44]	<0.001
Alcohol (units/week)	6.5 ± 9.7	0–42	0.86	[0.67, 1.11]	0.24
ALT (iu/L)	28.0 ± 19.9	11–175	1.28	[1.03, 1.60]	0.029
Fasting NEFA (mmol/L)	684.6 ± 208.9	286–1332	1.01	[0.77, 1.33]	0.94
AUC_{NEFA} (mmol/L min)	35435 ± 10353	19680–66180	1.35	[1.06, 1.74]	0.018
NEFA Suppression at 30 minutes (%)	38.6 ± 19.5	−11.2–81.9	0.75	[0.59, 0.96]	0.020
NEFA Suppression at 60 minutes (%)	67.8 ± 15.2	25.2–89.2	0.75	[0.58, 0.96]	0.023
Leptin (ng/mL)	15.3 ± 13.8	0.1–65.3	1.45	[1.002, 2.09]	0.049
Adiponectin (ug/mL)	7.7 ± 4.9	2.2–24.1	0.52	[0.40, 0.69]	<0.001
LAR (ng/ug)	3.0 ± 3.6	0.01–16.3	1.49	[1.05, 2.10]	0.026
Fasting glucose (mmol/L)	5.0 ± 0.5	4.1–6.4	1.49	[1.19, 1.85]	0.001
2-hour glucose (mmol/L)	7.3 ± 2.0	3.9–12.3	1.44	[1.15, 1.80]	0.002
HbA1c (%)	5.7 ± 0.3	4.9–6.4	1.11	[0.88, 1.39]	0.39
Fasting insulin (pmol/L)	64.7 ± 40.3	18.1–288	1.46	[1.13, 1.90]	0.005
HOMA-IR (%)	1.2 ± 0.7	0.4–5.0	1.50	[1.16, 1.92]	0.002
OGIS (mL min^{-1} m^{-2})	409.3 ± 62.7	223–548	0.56	[0.43, 0.72]	<0.001

Intrahepatic lipid was log transformed in all analyses, and then beta coefficients and confidence intervals were back transformed.
[a]Beta represents the ratio of geometric mean IHL per 1 standard deviation increase in relevant exposure. Therefore, standardized beta values > 1 represent a positive association, and values < 1 represent a negative association. All analyses are adjusted for age, gender, alcohol consumption, and MRI-derived visceral fat area (MRI VAT).
[b]These analyses are not adjusted for MRI VAT.
HOMA: homeostasis model assessment.
LAR: leptin : adiponectin ratio.
NEFA: nonesterified fatty acids.
OGIS: oral glucose insulin sensitivity.

Table 2: Associations between standardised measures of insulin sensitivity (as exposures) and measures of NEFA metabolism (as outcomes).

	Fasting NEFA			NEFA suppression at 30 minutes			NEFA suppression at 60 minutes		
	β	95% CI	*P*	β	95% CI	*P*	β	95% CI	*P*
Unadjusted									
zLAR	−17.8	[−75.8, 40.3]	0.54	−7.9	[−13.0, −2.8]	0.003	−6.0	[−10.0, −2.1]	0.003
zOGIS	10.4	[-46.4, 67.2]	0.72	9.1	[4.2, 13.9]	<0.001	5.6	[1.6, 9.5]	0.006
zHOMA	15.6	[36.2, 67.4]	0.55	−3.6	[−8.4, 1.2]	0.14	−1.8	−5.6, 2.1	0.35
Adjusted[a]									
zLAR	−31.6	[−105.3, 42.1]	0.39	−11.9	[−18.7, −5.1]	0.001	−7.3	[−12.6, -2.0]	0.008
zOGIS	−25.0	[−88.3, 38.3]	0.43	11.0	[5.4, 16.7]	<0.001	4.4	[−0.2, 9.1]	0.062
zHOMA	49.4	[−4.5, 103.3]	0.07	−3.6	[−9.1, 1.9]	0.19	0.02	[−4.2, 4.2]	0.99

[a]Adjusted for age, gender, MR VAT, and alcohol consumption.
NEFA: nonesterified fatty acids.
zHOMA: standardised homeostasis model assessment.
zLAR: standardised leptin : adiponectin ratio.
zOGIS: standardised oral glucose insulin sensitivity.

so the observation that NEFA suppression at 30 rather than 60 or 120 minutes is a stronger marker of insulin sensitivity is not unexpected.

The similar strengths of associations for LAR and OGIS with NEFA suppression are potentially interesting. (They occurred in opposite directions because the former measures insulin resistance while the latter measures insulin sensitivity.) Given that NEFA suppression is mediated by adipose tissue insulin sensitivity [6], we anticipated that LAR might be

Table 3: Associations between measures of insulin sensitivity and intrahepatic lipid before and after adjusting for NEFA suppression at 30 and 60 minutes.

	Model 1[a]			Model 2[b]			Model 3[c]		
	β	95% CI	P	β	95% CI	P	β	95% CI	P
LAR	1.11	[1.01, 1.21]	0.026	1.05	[0.96, 1.16]	0.29	1.06	[0.96, 1.16]	0.24
OGIS	0.99	[0.99, 1.00]	<0.001	0.99	[0.99, 1.00]	<0.001	0.99	[0.99, 0.99]	<0.001
HOMA-IR	1.78	[1.24, 2.55]	0.002	1.69	[1.20, 2.38]	0.003	1.79	[1.28, 2.49]	0.001

Intrahepatic lipid was log transformed in all analyses, and then beta coefficients and confidence intervals were back transformed.
[a]Beta represents the ratio of geometric mean IHL per 1 unit increase in relevant exposure. Association with intrahepatic lipid is adjusted for age, gender, MR VAT, and alcohol consumption.
[b]As per model 1 with additional adjustment for NEFA suppression at 30 minutes.
[c]As per model 1 with additional adjustment for NEFA suppression at 60 minutes.
HOMA: homeostasis model assessment.
LAR: leptin : adiponectin ratio.
OGIS: oral glucose insulin sensitivity.

more strongly associated with it than OGIS, the latter reflecting insulin-mediated glucose disposal primarily in skeletal muscle, but this was not the case. However, LAR has only previously been shown to correlate with whole body insulin sensitivity rather than fat tissue sensitivity specifically [13], and while leptin and adiponectin are derived exclusively from adipocytes rather than merely acting as markers of adipocyte inflammation, they may actively modulate insulin action in other tissues. The absence of an association between impaired NEFA suppression and HOMA (which is generally regarded as an index of hepatic insulin resistance) suggests that even though the liver is capable of disposal of free fatty acids [20], variations in hepatic insulin sensitivity have a relatively small impact on wholebody fatty acid disposal. Lastly, the finding that the inverse association between $NEFA_{AUC}$ and IHL was twice as strong as that for NEFA suppression at 30 or 60 minutes probably reflects the bidimensional nature of the AUC measure (see Figure 1), where fasting as well as postprandial NEFA levels have a multiplicative effect on this variable.

Our data indicate that excess body fat (particularly visceral fat), insulin resistance, and impaired suppression of NEFA levels after oral glucose loading are all directly associated with increased IHL in older individuals. In order to explore the mechanistic basis for the association between peripheral insulin resistance and NAFLD, we adjusted for NEFA suppression and found that this association was significantly attenuated for LAR but not for OGIS or HOMA. For these analyses, we also adjusted for central adiposity and chose the MRI-derived cross-sectional visceral fat area over other measures of fatness (BMI, body fat %, and waist circumference) because it was most strongly associated with liver fat content (Table 1). However, results were similar when these other measures of fatness were used (data not shown). So reduced suppression of fatty acids explains the association between LAR, but not OGIS or HOMA, and liver fat content. This suggests that while OGIS and LAR are both indices of whole body insulin sensitivity, adipocyte insulin resistance is reflected to a greater extent with LAR than with OGIS or HOMA. These results also suggest that there are other mechanisms apart from impaired NEFA suppression linking insulin resistance and NAFLD, such as dysregulated de novo lipogenesis and hepatocyte endoplasmic reticulum stress [21].

This study has a number of important strengths. Very detailed anthropometric and metabolic characterisation was conducted in each participant, and results were consistent across different measures of body fatness and insulin sensitivity. MR spectroscopy is the most robust noninvasive method for quantifying IHL. Rather than comparing categories of steatosis, body fatness, or glycaemic status, as many studies do, all our measures are continuous and represent the distributions in a relatively healthy cohort of older white participants. The study also has some limitations. It is a post-hoc analysis of data from a subgroup of individuals who were willing to participate in an exercise trial. All participants in the trial were White. Thus, our results may not be generalisable to all older people or those who would be less amenable to an exercise intervention, for whatever reason. Also, only 70% of people who enrolled in the trial had MR imaging at baseline, while others were unwilling or were too large for the scanner, which may have introduced bias.

Nonetheless, the 42% prevalence of NAFLD was higher than we anticipated, and there was a substantial level of metabolic disturbance in this cohort, particularly in relation to the number of individuals with abnormal glucose metabolism during the OGTT. It is important to note that these abnormalities were only detected through participation in the study and were not diagnosed prior to it (and so were not "prevalent" as such). All of these individuals volunteered to participate in a 12-week exercise intervention. Diabetes was one of several exclusion criteria. Nonetheless, three of the 70 individuals (4.3%) had newly diagnosed type 2 diabetes at entry to the study, while a further 27 (38.6%) had abnormal glucose metabolism. None of these had symptoms of hyperglycaemia, nor were they on treatment for it at the time of testing. Therefore, we felt it is appropriate to include them in the analysis. These participants were "apparently

healthy," and within a cohort of this age, a certain level of undiagnosed metabolic disease, be it diabetes or liver steatosis, is to be expected, so to be able to quantify this so precisely in the paper contributes to the novelty of our findings. So our observation too that impaired NEFA suppression does mediate the association between whole body insulin resistance, measured with LAR and liver steatosis.

5. Conclusions

In our experience, there is a widely held perception amongst scientists and clinicians in the field of metabolism that fasting NEFA levels are strongly positively associated with insulin resistance. However, in conducting a formal comparison of the relative strengths of the associations between fasting versus suppressed NEFA levels, we have confirmed that the degree of NEFA suppression is far more strongly associated with indices of insulin resistance, namely, OGIS and LAR, and is thus important from a clinical and pathophysiological point of view. We have been careful to take account of confounding factors such as age [22], sex [8], and other factors likely to influence these associations such as alcohol consumption. We believe that the relatively good health of the participants in this study makes the findings described above, particularly in relation to the high prevalence of NAFLD, even more novel and compelling.

Abbreviations

DEXA: Dual energy X-ray absorptiometry
HOMA: Homeostasis model assessment
HPAT: Hertfordshire Physical Activity Trial
IHL: Intrahepatic lipid
LAR: Leptin : adiponectin Ratio
NAFLD: Nonalcoholic fatty liver disease
OGIS: Oral glucose insulin sensitivity
VAT: Visceral adipose tissue.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

Authors' Contribution

Francis M. Finucane contributed to study design, data acquisition, analysis and interpretation, and writing the paper. Stephen J. Sharp contributed to study design, data analysis and interpretation, and writing the paper. Mensud Hatunic contributed to data interpretation and writing the paper. Alison Sleigh contributed to study design, data acquisition and analysis, and writing the paper. Ema De Lucia Rolfe contributed to study design, data acquisition and analysis, and writing the paper. Avan Aihie Sayer contributed to study design, data acquisition, and writing the paper. Cyrus Cooper contributed to study funding, design, data acquisition, and writing the paper. Simon J. Griffin contributed to study funding, design, data acquisition, analysis and interpretation, and writing the paper. David B. Savage contributed to study design, data acquisition, analysis and interpretation, and writing the paper. Nicholas J. Wareham contributed to study funding, design, data acquisition, analysis and interpretation, and writing the paper.

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Research Article

Major Histocompatibility Class II Pathway Is Not Required for the Development of Nonalcoholic Fatty Liver Disease in Mice

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Single-nucleotide polymorphisms within major histocompatibility class II (MHC II) genes have been associated with an increased risk of drug-induced liver injury. However, it has never been addressed whether the MHC II pathway plays an important role in the development of nonalcoholic fatty liver disease, the most common form of liver disease. We used a mouse model that has a complete knockdown of genes in the MHC II pathway ($MHCII^{\Delta/\Delta}$). Firstly we studied the effect of high-fat diet-induced hepatic inflammation in these mice. Secondly we studied the development of carbon-tetra-chloride- (CCl_4-) induced hepatic cirrhosis. After the high-fat diet, both groups developed obesity and hepatic steatosis with a similar degree of hepatic inflammation, suggesting no impact of the knockdown of MHC II on high-fat diet-induced inflammation in mice. In the second study, we confirmed that the CCl_4 injection significantly upregulated the MHC II genes in wild-type mice. The CCl_4 treatment significantly induced genes related to the fibrosis formation in wild-type mice, whereas this was lower in $MHCII^{\Delta/\Delta}$ mice. The liver histology, however, showed no detectable difference between groups, suggesting that the MHC II pathway is not required for the development of hepatic fibrosis induced by CCl_4.

1. Introduction

Major histocompatibility class II (MHC II) pathway plays an important role in immune function. The molecules of MHC II are expressed on the surface of antigen-presenting cells such as macrophages, B cells, and dendritic cells [1]. Once the processed antigen loaded onto MHC II molecules is presented on the surface of the cells, it promotes the CD4+ helper T cell recognition. This results in an immune response including the production of inflammatory cytokines [2]. Although the importance of the pathway has been widely studied in the immune processing, its specific roles on hepatic inflammation and fibrosis have not been clearly understood.

A genome-wide association study identified single-nucleotide polymorphisms (SNPs) in the MHC II pathway in lumiracoxib-treated patients that developed a liver injury [3]. The alleles of the genes (HLA-DRB1, 5, -DQB1, and -DQA1) had a strong association with elevated plasma liver enzymes in patients that developed the liver injury after the lumiracoxib treatment. Lumiracoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, has been used for osteoarthritis and acute pain treatment [4, 5]. The use of this COX-2 inhibitor is correlated with an increased risk of cardiovascular events and an acute hepatotoxicity [6, 7]. However whether the MHC II pathway has been involved in the mechanism of these diseases has not been clarified.

A similar observation has been reported with a treatment of amoxicillin-clavulanate, an antimicrobial agent [8, 9]. Again the SNPs in the region around HLA-DRB1 and HLA-DQB1 showed a strong association with the drug-induced liver injury. Interestingly, the alleles in HLA-DQB1 locus were also strongly associated with primary biliary cirrhosis [10], which is the most common autoimmune liver disease. During the development of biliary cirrhosis, T lymphocytes play an important role [11, 12], and a link between

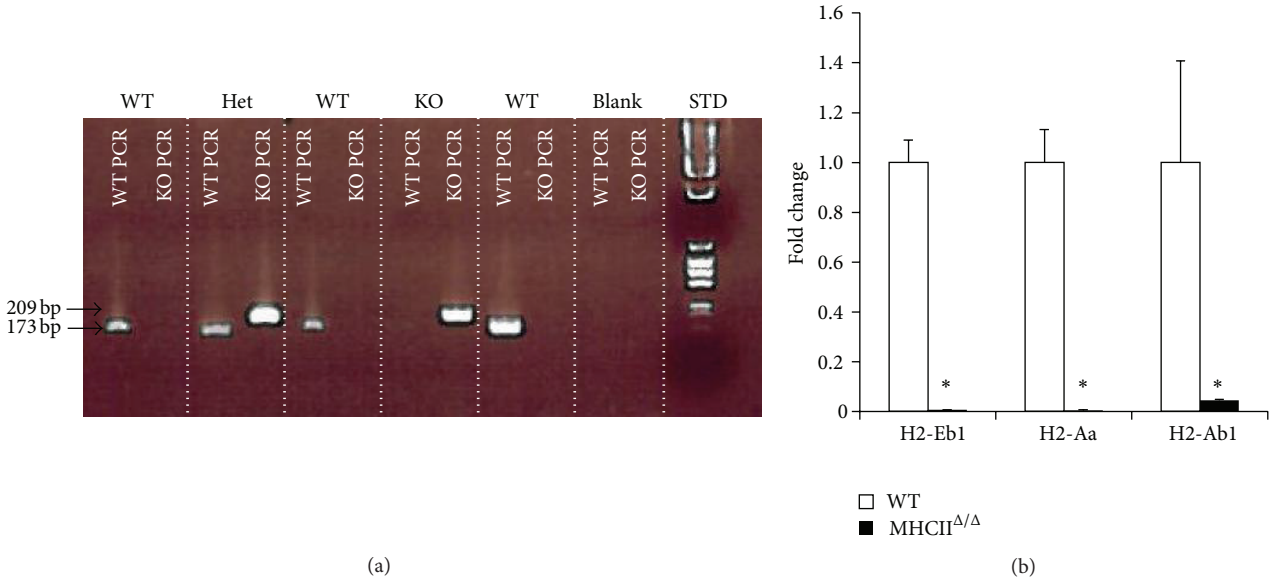

Figure 1: MHCII gene genotyping and hepatic expression in wild-type and MHCII$^{\Delta/\Delta}$ mice. (a) Two different PCRs were performed for the detection of wild-type (WT PCR) and knockout (KO PCR: MHCII$^{\Delta/\Delta}$) fragments; 173 bp for a WT band and 209 bp for a KO band. Therefore heterozygous (Het) presents both bands. (b) Gene expression of MHC II genes (H2-Eb1, -Aa, -Ab1) in liver samples by real-time RT-PCR. The expression was normalized by β2 microglobulin and compared to the wild-type. Open bars represent wild-type and closed bars MHCII$^{\Delta/\Delta}$ mice (n = 10–14/group). Data are presented as mean ± SEM. *Significantly different from wild-type mice, $P < 0.05$ (Student t-test).

the T lymphocytes hyperactivity and drug-induced liver injuries has been recently suggested [13]. In addition, several studies have demonstrated that the genes in the MHC II pathway were significantly upregulated in porcine-serum-induced hepatic fibrosis in rats [14, 15]. These facts imply a strong influence of the MHC II pathway on the susceptibility to develop liver diseases.

Nonalcoholic fatty liver disease (NAFLD) is one of the major liver diseases in industrialized countries. Data are suggesting a strong increase of NAFLD prevalence in the next decades [16, 17]. The disease consists of a diverse spectrum of liver pathologies, starting by hepatic steatosis and then steatohepatitis, a state of hepatic inflammation [18]. Then it progresses toward hepatic fibrosis, cirrhosis, and hepatic carcinoma. Although some molecular pathways that lead to hepatitis in NAFLD can also be activated in the drug-induced liver injuries, the implication of MHC II pathway has not been clearly understood in NAFLD model. Given the repeated reports on the association between alleles on MHC II genes and the susceptibility to a liver inflammation [3, 8–10], this pathway might play an important role in the development of NAFLD.

In the present study, we addressed whether the MHC II pathway might be required for hepatitis development and fibrosis formation. To this end, we chose to use a mouse model lacking all conventional genes in MHC II pathway [19]. The entire MHC II region (80 kb) was deleted, resulting in the removal of the genes encoding the MHC II pathway (H2-Aβ, -Aα, -Eβ, -Eβ2, and -Eα). The mouse genes H2-Aβ and -Eβ have the closest homology to the human HLA-DQB1 gene. These mice are viable and fertile without major anatomical or physiological abnormalities [19]. We have studied whether these mice were protected against a high-fat diet-induced hepatitis and a chemical-induced hepatic fibrosis.

2. Research Design and Method

2.1. Animals, Diet, and Chemicals. B6;129S2-*H2*$^{dlAb1\text{-}Ea}$/J (strain 003584, MHCII$^{\Delta/\Delta}$) mice in which the 80 kb of MHC II region is deleted were purchased from Jackson Laboratories (Bar Harbor, ME, USA). These mice were bred with C57B6/J mice and the F2 generation was crossbred to obtain MHCII$^{\Delta/\Delta}$ and wild-type mice. These mice were housed in ventilated cages at an animal facility of the University of Lausanne with 12-hour light and dark cycles and free access to food and water. Five-week-old male mice were subjected to high-fat diet (D12451, Research diets Inc., New Brunswick, NJ, USA) for 4 months. The dietary composition of the high-fat diet was carbohydrates 35%, fat 45%, and protein 20%. Dietary protein was originated from casein, and fat was mainly from lard. All animal procedures used in this study were approved by the Swiss cantonal veterinary service.

Carbon tetrachloride (CCl_4), chloroform, methanol, EDTA, sirius red, and aprotinin were purchased from Sigma-Aldrich (Munich, Germany). Kits for measuring plasma alanine transaminase (ALT), aspartate transaminase (AST), and triglycerides (TG) were purchased from Wako Chemicals (Neuss, Germany). Hematoxylin & eosin was purchased from Merck (Geneva, Switzerland).

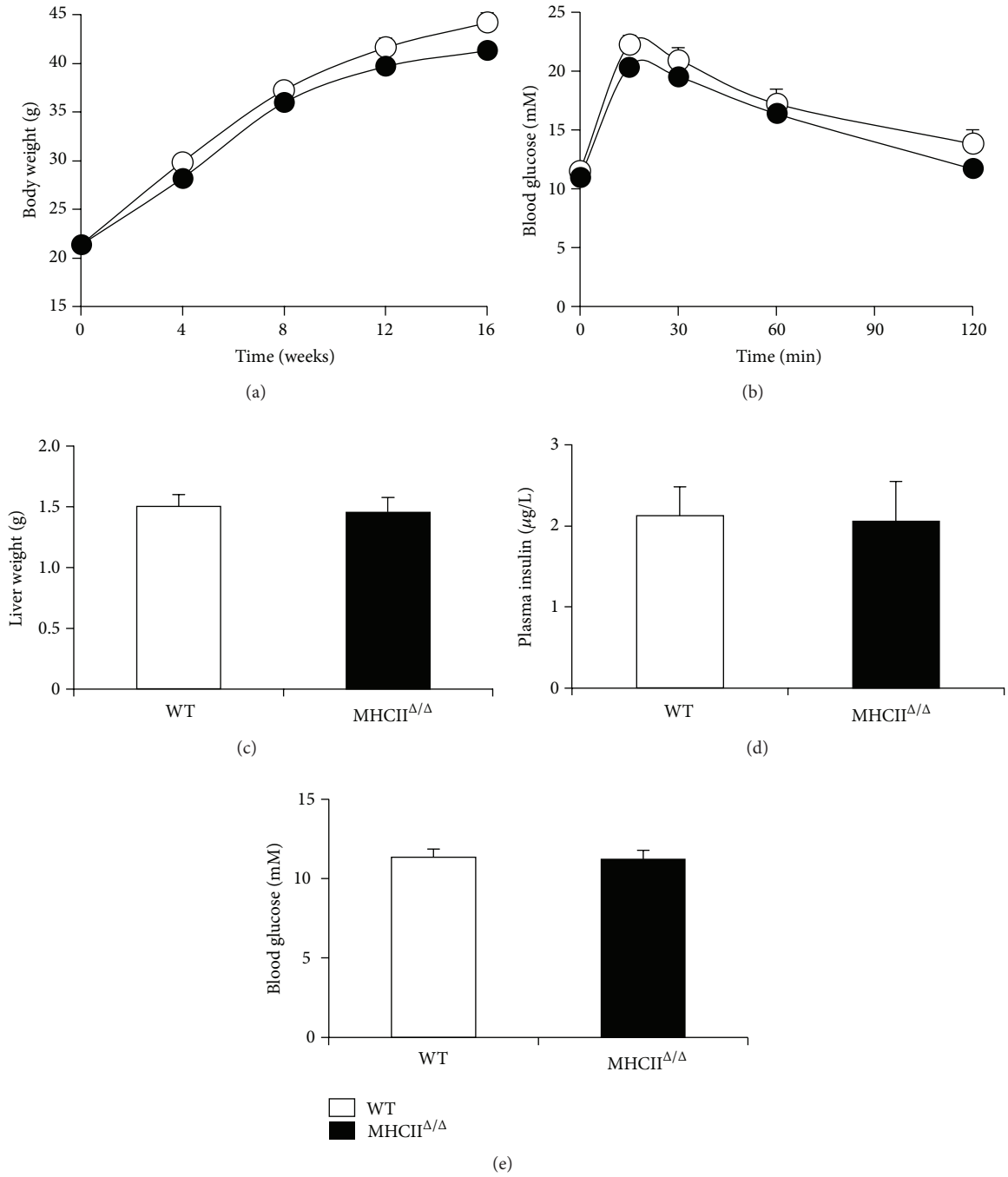

Figure 2: Effect of high-fat diet on metabolic parameters in wild-type and MHCII$^{\Delta/\Delta}$ mice. Open circle/bars represent wild-type and closed circle/bars MHCII$^{\Delta/\Delta}$ mice (wild-type: $n = 25$, MHCII$^{\Delta/\Delta}$: $n = 20$). Data are presented as mean ± SEM. (a) Body weight gain during 4-month diet. (b) Glucose tolerance test was performed around the 15th week of the intervention. (c) Liver weight at 16th week. (d) Fasting plasma insulin concentration at 16th week. (e) Fasting blood glucose concentration at 16th week.

2.2. High-Fat Diet Experiment. Five-week-old male MHCII$^{\Delta/\Delta}$ mice and wild-type mice were assigned into the high-fat diet described above. Body weight was measured every month. A glucose tolerance test was performed around the 15th week of the intervention. Mice were fasted for 4 hours and a solution of 1 g of glucose per kg of mouse was injected intraperitoneally. Glycemia was monitored at 0, 15, 30, 60, and 120 min using a glucometer (Bayer, Zurich, Switzerland).

At the end of the 4-month experiment, mice were fasted for 4 hours and glycemia was measured by a glucometer. Blood was collected by an intracardiac puncture and placed into a tube containing EDTA and aprotinin (2 mM and 0.1-0.2 TIU, respectively) on ice. The plasma was then separated

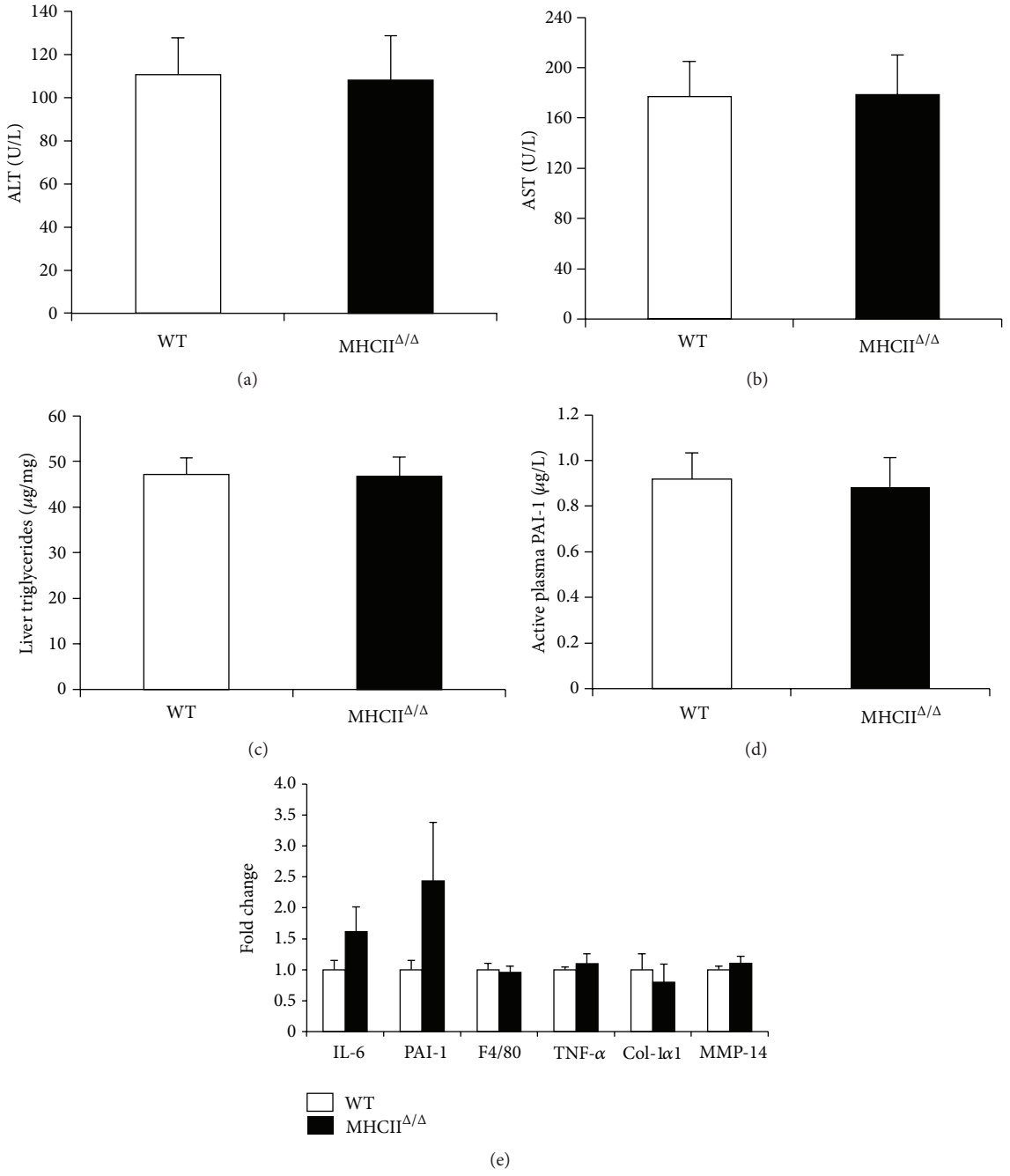

Figure 3: Effect of high-fat diet on liver enzymes, lipids, and inflammatory markers in wild-type and MHCII$^{\Delta/\Delta}$ mice. Open bars represent wild-type and closed bars MHCII$^{\Delta/\Delta}$ mice (wild-type: $n = 25$, MHCII$^{\Delta/\Delta}$: $n = 20$). Data are presented as mean ± SEM. (a) Plasma ALT level at 16th week. (b) Plasma AST level. (c) Liver triglyceride content at 16th week. (d) Fasting active plasma PAI-1 concentration at 16th week. (e) Gene expression related to inflammation and fibrogenesis in the liver at 16th week.

by centrifugation and stored at −80°C until analysis. Liver and epididymal adipose tissues were harvested and weighed. The organs were flash-frozen into liquid nitrogen and stored at −80°C until analysis.

2.3. CCl_4 Treatment. To study the development of hepatic fibrosis, 8-week-old wild-type and MHCII$^{\Delta/\Delta}$ male mice were treated by 1.25 μL/g body weight CCl_4 (25% in sunflower oil) twice a week during 4 weeks. Another set of mice was treated by sunflower oil as control vehicle at the same time. Twenty hours following the last treatment, mice were fasted for 4 hours and anesthetized for a cardiac puncture. After sacrifice, blood and liver were kept for further analysis. Fresh liver pieces were put into 4% paraformaldehyde solution during

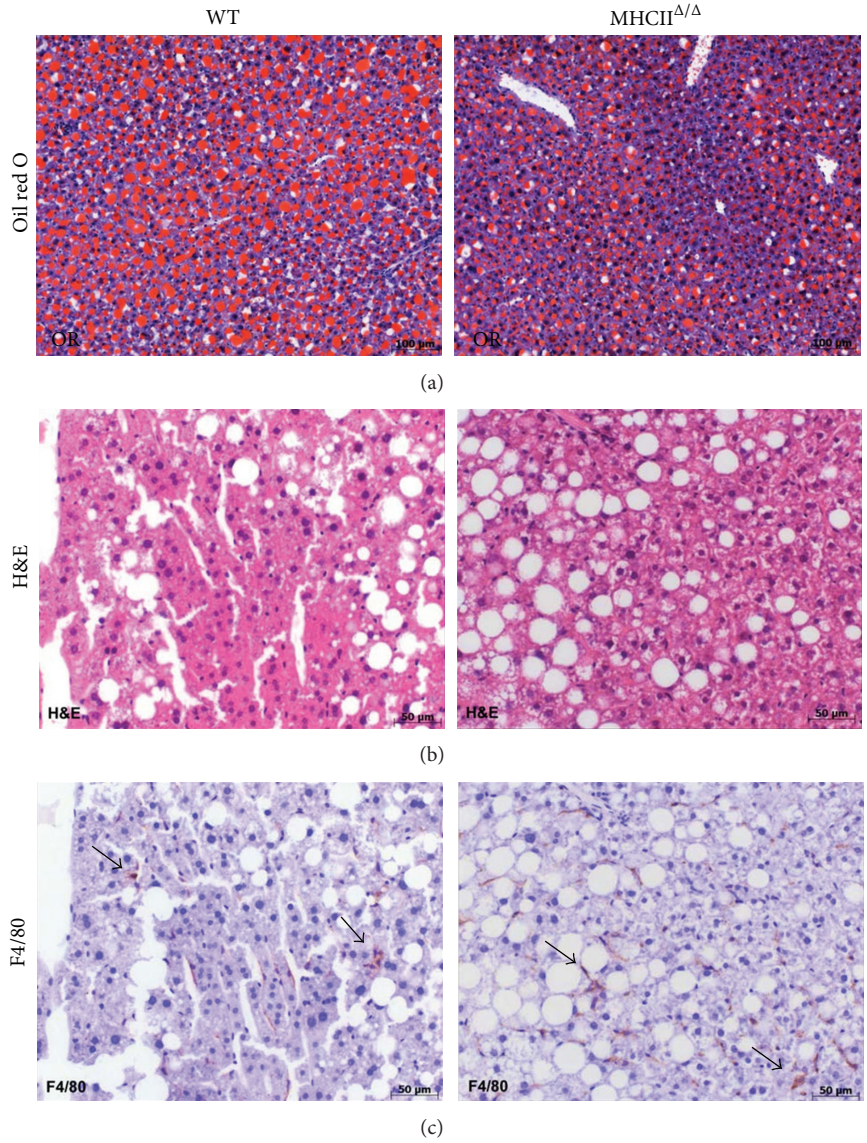

Figure 4: Effect of high-fat diet on liver histology in wild-type and MHCII$^{\Delta/\Delta}$ mice. (a) Oil red O staining (red color represents the lipid accumulation). (b) Hematoxylin and eosin (H&E) staining. (c) F4/80 immunohistochemistry (macrophages were stained in brown: arrows).

4 hours. After several washings with PBS, liver samples were dehydrated and parafinated for histological analysis.

2.4. Histology. Parafinated liver samples were sliced (4 μm) and stained according to a standard technique with hematoxylin and eosin (H&E) using routine methods. To study the fibrosis formation, 0.1% sirius red was used to stain collagen I, III, and bile pigment using a protocol described elsewhere [20]. For the histological samples, photos were taken with an AXIO Imager M1 with fluo-Axiocam MRm and color Axiocam MRc cameras (Carl Zeiss AG, Oberkochen, Germany). Images were treated by Axiovision release 4.8.2. We then quantified the fibrotic area by assessing the ratio of the red-stained area (fibrosis) to the total area, the vascular luminal area being subtracted if present, using Photoshop (Adobe Systems, Mountain View, USA) in five images per section of the sample (10 x magnification).

Immunohistochemistry by glial fibrillary acidic protein (GFAP) stain was performed by the subsequent incubations of the slices with rabbit anti-GFAP primary antibody (Dako, Glostrup, Denmark) diluted in normal goat serum for overnight at 4°C, then with goat anti-rabbit coupled with Alexa Fluor 568 (Life Technologies, Carlsbad, USA) for 30 minutes in the dark at room temperature. Immunostained sections were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI), embedded with mowiol and analyzed using the Zeiss microscope.

2.5. Plasma Parameters and Liver Lipids Analysis. Total plasma ALT and AST were measured by the Roche/Hitachi

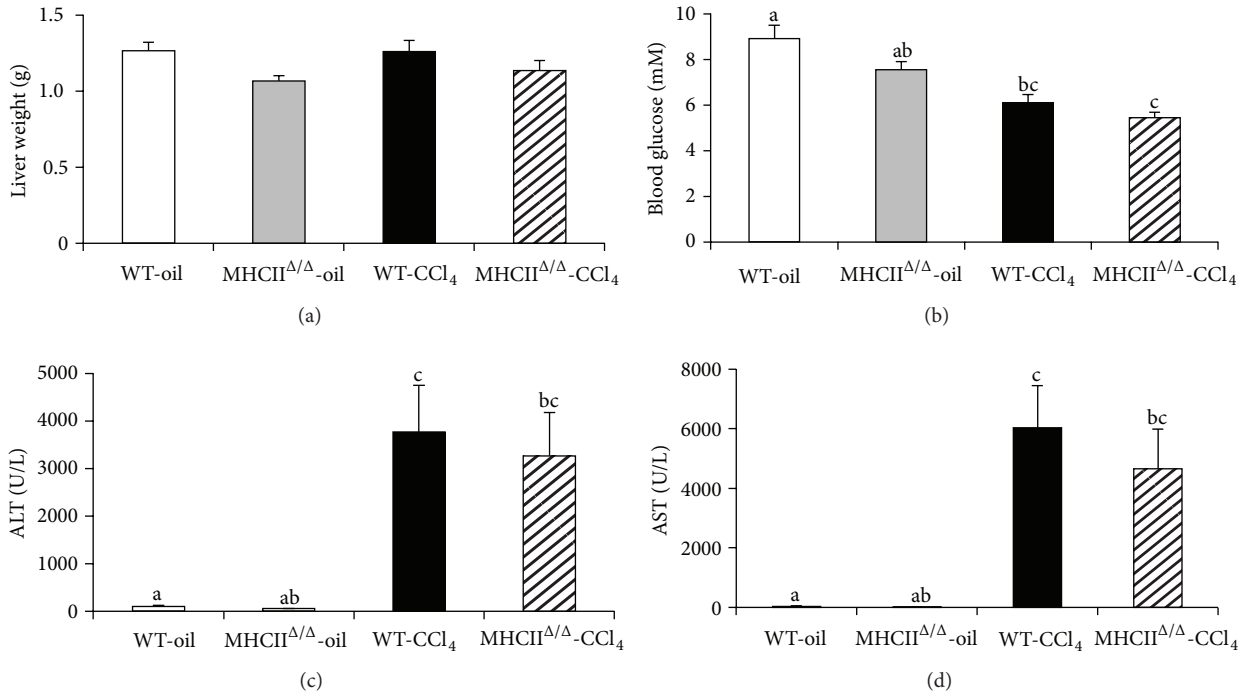

Figure 5: Effect of 4-week CCl_4 treatment on liver and blood glucose in wild-type and MHCII$^{\Delta/\Delta}$ mice. Open bars represent oil-treated wild-type (WT-oil), closed bars CCl_4-treated wild-type (WT-CCl_4), gray bars oil-treated MHCII$^{\Delta/\Delta}$ mice, and semiclosed bars CCl_4-treated MHCII$^{\Delta/\Delta}$ mice (wild-type oil: $n = 7$, MHCII$^{\Delta/\Delta}$ oil: $n = 5$, wild-type CCl_4: $n = 11$, MHCII$^{\Delta/\Delta}$ CCl_4: $n = 9$). Data are presented as mean ± SEM. (a) Liver weight. (b) Fasting blood glucose decreased after the CCl_4 treatment in both groups. (c), (d) Strong increase in fasting plasma ALT and AST was observed after the CCl_4 treatment. $^{a,b,c}P < 0.05$, ANOVA and Tukey Kramer HSD test. Different letters indicate the significant difference between groups.

912 instrument with the commercial kits mentioned earlier. Insulin was measured by a Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden). Plasma active Plasminogen activator inhibitor-1 (PAI-1) was also measured by an ELISA kit (Molecular Innovations Inc., Novi).

From the harvested liver samples, total lipids were extracted using a modified Folch method [21, 22]. For the measurement of hepatic TG content, total lipid extract was subjected to SPE columns (Interchim, Montluçon, France) to separate TG [23, 24]. TG were then mixed with a chloroform-triton X (1%) solution and dried under N_2 gas. TG were thus dissolved into water and the content of TG was measured by the use of the Wako Chemical kit.

2.6. Genotyping and Real-Time Polymerase Chain Reaction (PCR). Ear DNA was extracted by the hotSHOT protocol [25]. Genotyping was performed according to Jackson laboratory's protocol (http://jaxmice.jax.org/strain/003584.html). Briefly, 2 sets of primers were used for the mutated gene (oIMR1020: 5′-Cgg AAg TgC TTg ACA TTg g-3′, oIMR1021:5′-gTA TTg ACC gAT TCC TTg Cg-3′) and wild-type gene (oIMR1273; 5′-AAC CTT CAg gAT CTg TgA TCC-3′, oIMR1274; 5′-gTg gCT gTT gCC TTA AgA CC-3′). After PCR cycles, samples were loaded onto 2% agarose gels and the mutated band (209 bp) as well as the wild-type band (178 bp) was monitored.

Total RNA was extracted from tissues according to the phenol-chloroform extraction protocol in Tri Reagent (Molecular Research Center, Inc., Cincinnati, USA) [26]. cDNA was created by a reverse transcription of 2 μg of total RNA using Superscript II Transcriptase from Invitrogen (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. Real-time RT-PCR was performed on Applied Biosystems' 7000 Sequence Detection System (Life Technologies, Carlsbad). Twenty-time diluted cDNA samples and 0.3 μM forward and reverse primers (Microsynth AG, Balgach, Switzerland) in a final 10 μL volume were reacted in the following PCR cycle conditions: 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Each sample was analyzed in duplicate using the DeltaDeltaCt method [27]. β2 microglobulin (β2M) was used as a housekeeping gene to normalize the expression of each gene.

2.7. Statistics. All data are shown in mean ± standard error of the mean (SEM). Two groups were compared by Student t-test. Four groups were compared by one-way analysis of variance (ANOVA) and once it reached the significance ($F < 0.05$), a post hoc test (Tukey Kramer HSD test) was performed. The statistical analyses were performed by use of the JMP software (SAS Institute Inc., Cary).

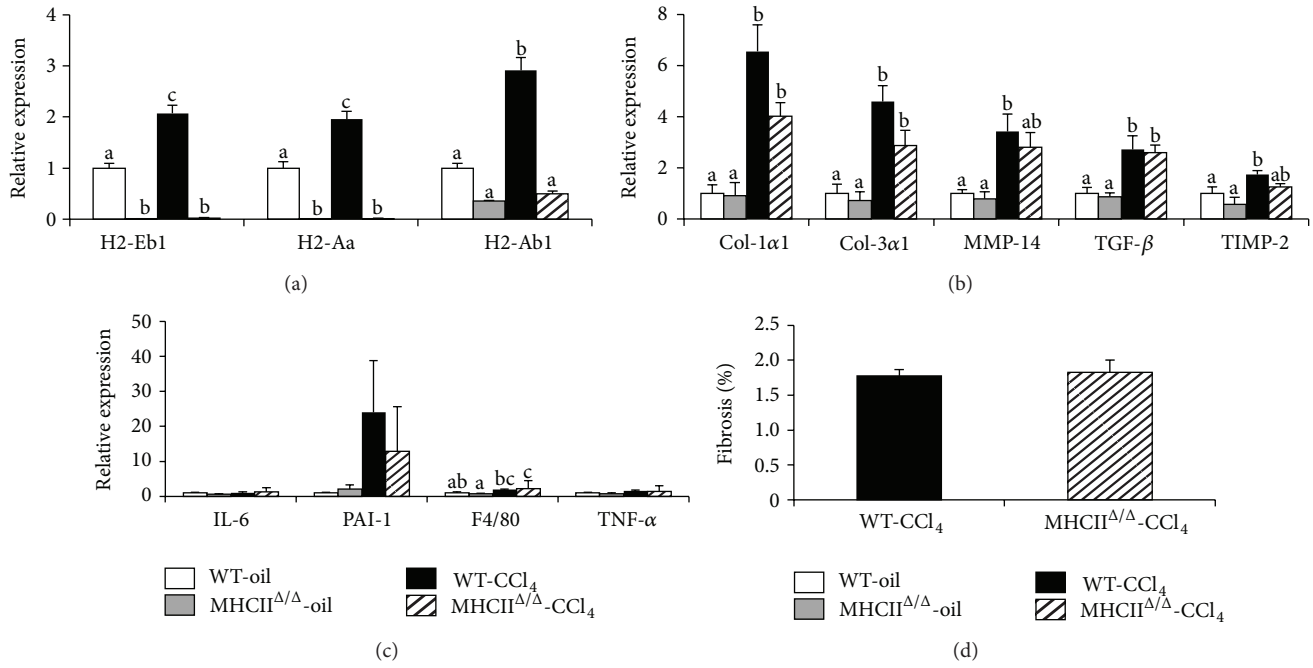

Figure 6: Effect of 4-week CCl_4 treatment on hepatic gene expression and fibrosis in wild-type and $MHCII^{\Delta/\Delta}$ mice. Open bars represent oil-treated wild-type (WT-oil, $n = 7$), closed bars CCl_4-treated wild-type (WT-CCl_4, $n = 11$), gray bars oil-treated $MHCII^{\Delta/\Delta}$ mice ($MHCII^{\Delta/\Delta}$-oil, $n = 5$), semiclosed bars CCl_4-treated $MHCII^{\Delta/\Delta}$ mice ($MHCII^{\Delta/\Delta}$-CCl_4, $n = 9$). Data are presented as mean ± SEM. (a) MHC II gene expression. (b) Gene expression related to fibrosis formation. (c) Gene expression related to inflammation. (d) No difference in fibrosis (%) between groups treated by CCl_4. The percentage was determined based on the sirius red staining (5 pictures/mice, WT-CCl_4: $n = 5$, $MHCII^{\Delta/\Delta}$-CCl_4: $n = 8$). $^{a,b,c}P < 0.05$, ANOVA and Tukey Kramer HSD test. Different letters indicate the significant difference between groups.

3. Results

3.1. Validation of the Mouse Model. The $MHCII^{\Delta/\Delta}$ mice were originally created by the laboratory of Dr. Christophe Benoist (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). These mice lack the major genes of MHC II pathway and present very low counts of CD4+ T lymphocytes in the thymus and spleen [19]. The detailed genetic modification and their phenotype have been published [19]. These mice have been backcrossed with C57B6/J strain at Jackson laboratory. After purchasing the mice from Jackson laboratory, we crossed them with C57B6/J mice to obtain control wild-type animals. In this model, the PCR for detecting the knockout allele showed a clear band at 209 bp. The quantitative PCR analysis showed no or very low expression of the genes of the MHC II pathway (Figure 1).

3.2. Effect of High-Fat Diet on Inflammatory Status in $MHCII^{\Delta/\Delta}$ Mice. Both wild-type and $MHCII^{\Delta/\Delta}$ mice similarly gained body weight after 4-month high-fat diet (Figure 2). Liver weight was also similar in both groups. Plasma glucose and insulin concentrations were comparable between groups. As expected, both groups had similar glucose tolerance after the long-term high-fat diet. The liver TG content was also comparable between groups (Figure 3). Oil red O staining confirmed this result (Figure 4). Plasma ALT and AST levels were equally high after the high-fat diet in two groups (Figure 3), suggesting similar liver functions after the high-fat diet.

To study the inflammatory status of the liver after the long-term high-fat diet feeding, we have performed H&E staining and F4/80 for macrophages staining. We observed positive markers of F4/80 and similar lipid droplet morphology in both groups (Figure 4). The expression of genes related to inflammation such as IL-6, TNF-α, PAI-1, and F4/80 was not different between groups (Figure 3). The mRNA levels of fibrosis markers such as collagen type 1α1 (Col-1α1) and matrix metalloproteinase-14 (MMP-14) were also comparable. We also measured active PAI-1 concentration in the plasma (Figure 3). Again no difference was observed between wild-type and $MHCII^{\Delta/\Delta}$ mice. All together, blocking the MHC II pathway did not affect the hepatic inflammation induced by the high-fat diet in mice.

3.3. Effect of CCl_4 Injection on the Formation of Hepatic Fibrosis in $MHCII^{\Delta/\Delta}$ Mice. We next studied the development of hepatic fibrosis. To this end, we treated wild-type and $MHCII^{\Delta/\Delta}$ mice with CCl_4 during 4 weeks. The treatment resulted in a significant decrease of glycemia and a significant increase of ALT and AST in both groups (Figure 5). No difference in liver weight was observed. The CCl_4 injections also highly induced the expression of MHC II genes in wild-type mice, while $MHCII^{\Delta/\Delta}$ mice had no induction

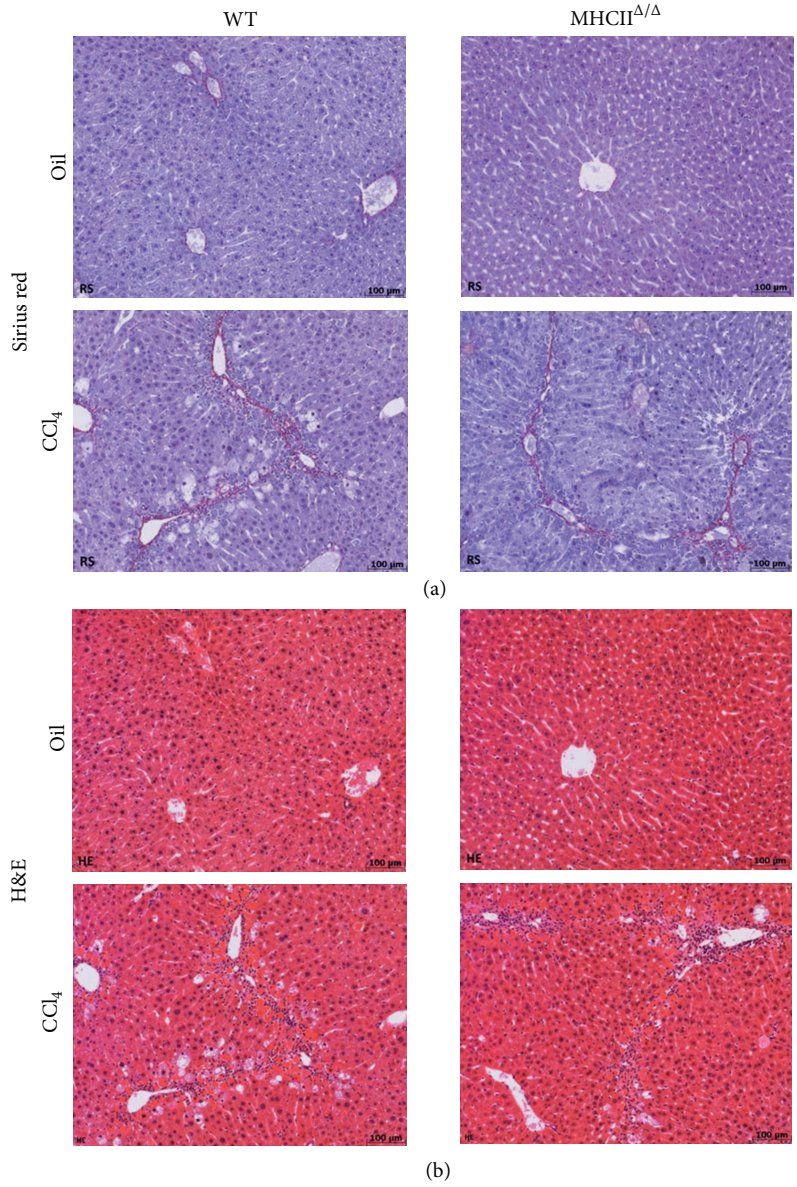

Figure 7: Effect of 4-week CCl_4 treatment on liver histology in wild-type and MHCII$^{\Delta/\Delta}$ mice. (a) Sirius red staining for collagen. The red staining represents fibrotic area. (b) Hematoxylin and eosin (H&E) staining.

of these genes, as expected (Figure 6). Genes related to the fibrosis formation such as Col-1α1, Col-3α1, and transforming growth factor-β (TGF-β) significantly increased in both groups after the CCl_4 injections compared to the oil-injected groups. The genes such as MMP-14 and tissue inhibitor of metalloproteinase-2 (TIMP-2) were also highly induced after CCL_4 treatment in wild-type mice: however, the induction was somewhat blunted in MHCII$^{\Delta/\Delta}$ mice.

The liver histology was analyzed using sirius red staining for fibrosis formation. No positive staining was detected in oil-treated groups. The level of positive staining in CCl_4-treated livers was quantified using Photoshop. We detected a comparable level of fibrosis between groups treated by CCl_4 (Figures 6 and 7). H&E staining also showed inflammatory signs in the liver treated by CCl_4 (Figure 7). Again, no remarkable difference was observed between wild-type and MHCII$^{\Delta/\Delta}$ mice. The staining by F4/80 showed a remarkable infiltration of macrophages in the livers of mice treated by CCl_4, but, again, no difference was observed between wild-type and MHCII$^{\Delta/\Delta}$ mice (Figure 8). We further stained stellate cells by GFAP. Interestingly we detected only one positive staining out of 13 in wild-type mice while we found 7 positive stainings out of 9 samples in MHCII$^{\Delta/\Delta}$ mice (Figure 9).

4. Discussion

The present study demonstrated that the MHC II pathway is not implicated in the development of hepatitis when induced

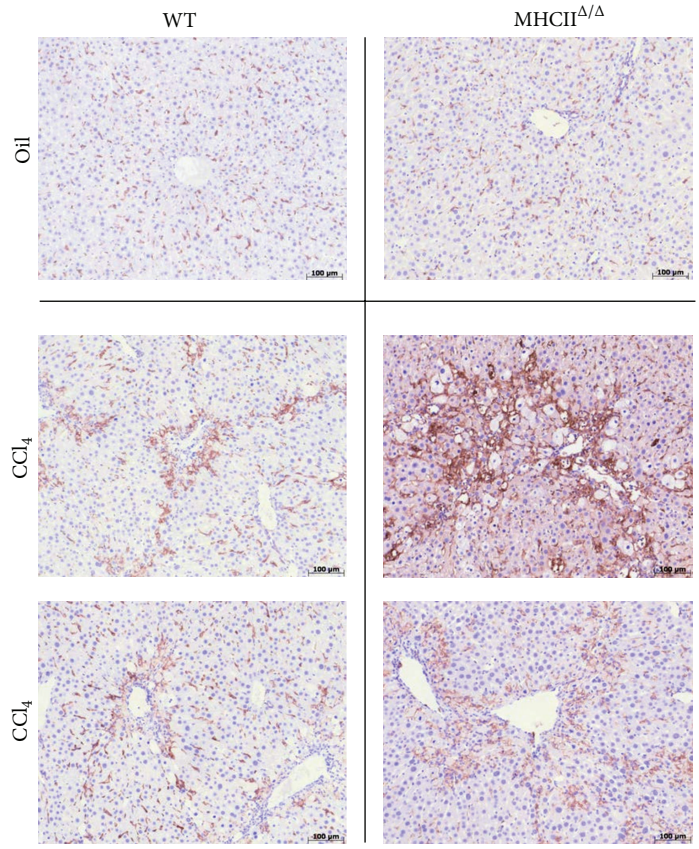

Figure 8: Effect of 4-week CCl_4 treatment on macrophage staining in wild-type and MHCII$^{\Delta/\Delta}$ mice. Macrophage staining (F4/80) showed a strong inflammation in both groups treated by CCl_4.

by a long-term high-fat diet. We had taken the approach of the high-fat diet to mimic our obesogenic lifestyle that is known to contribute to the nonalcoholic steatohepatitis (NASH). It is also strongly supported that feeding laboratory animals with high-caloric diets such as a high-fat diet can induce NASH [28–30]. As proposed in the pathophysiology of NASH, the "two-hit theory" could also explain the progression of hepatic steatosis to NASH in our experimental model. The first hit is an infiltration of fat into the hepatocytes (hepatic steatosis) and the second hit is characterized by an infiltration of immune cells such as monocytes and lymphocytes because of abnormal oxidative stress [31, 32]. Our mice having undergone a 4-month high-fat diet presented a high amount of fat content in the liver in both groups (first hit). Extensive high-fat diet feeding then resulted in a comparable increase of hepatic inflammation/damage in two groups, judged by macrophage infiltration (second hit). These data suggest that the long-term high-fat diet induced both hepatic steatosis and NASH; however, blocking the MHC II pathway had no influence on the development of hepatic steatosis and NASH in these mice.

Our long-term high-fat diet also increased the level of ALT and AST in both groups compared to the normal range of ALT and AST generally observed in chow-fed mice (15–80 U/L and 40–120 U/L, respectively, in our laboratory measurements). This result strongly suggests that the modification in MHC II gene expression affected neither ALT nor AST levels, consistent to their comparable liver histology between groups. It has been, however, suggested that some alleles in MHC II pathway were associated with a severity of NAFLD and ALT, but not AST levels, in a Turkish population [33]. In this study, the investigators could not assess a presence of NASH in the population. To our knowledge, this study is the only report suggesting an association between the HLA allele and NAFLD in general population. Many other studies have shown a connection between the HLA allele and plasma ALT or NASH severity in hepatitis C patients or in patients with a drug-induced liver injury [3, 6, 8, 9, 34, 35]. Therefore, MHC II pathway might have a bigger importance to the development of NASH caused by viral infections or drugs than by diet-induced NAFLD.

Secondly, we focused on the chemically induced fibrosis in mice lacking all conventional MHC II genes. As mentioned before, some HLA alleles are associated with an increased susceptibility to develop a drug-induced liver injury. The CCl_4 treatment strikingly increased the plasma level of ALT, AST, and the degree of hepatic fibrosis in both genotypes. The genes of MHC II pathway were also highly induced by CCl_4 treatment in wild-type mice, indicating the upregulation of the pathway upon the treatment. Despite the lack of the upregulation of the pathway, the MHCII$^{\Delta/\Delta}$ mice equally developed a hepatic fibrosis. These data strongly indicate

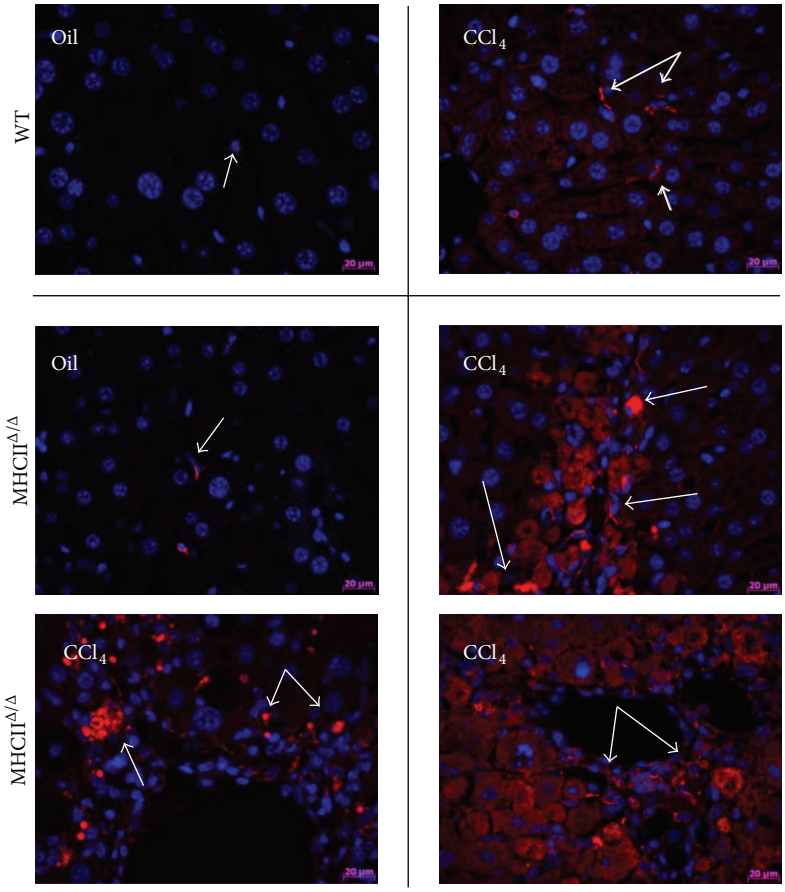

Figure 9: Effect of 4-week CCl_4 treatment on GFAP staining in wild-type and MHCII$^{\Delta/\Delta}$ mice. Glial fibrillary acidic protein (GFAP; white arrows) was stained in red. Increased positive staining was observed in CCl_4-treated MHCII$^{\Delta/\Delta}$ mice. Blue: DAPI staining.

that the MHC II pathway is not required for the development of hepatic fibrosis, at least in the mouse model of CCl_4-induced hepatic fibrosis.

Although the major genes implicated in the formation of fibrosis, namely, Col-1α1, Col-3α1, and TGF-β were similarly upregulated in the groups of mice treated by CCl_4, the induction of some genes such as TIMP-2 and MMP-14 tended to be lower in the MHCII$^{\Delta/\Delta}$ mice compared to the wild-type mice. TIMP-2 expression has been observed during the early stages of fibrogenesis induced by a porcine serum [36]. We thought that the deletion of MHC II genes might have affected the progression of fibrosis at an earlier time point. In our study, we had tested different periods of injection of CCl_4 (2-, 4-, 6-, and 8-week treatment) in these mice. In any condition tested, we did not observe a significant difference in the gene expression related to the fibrosis formation (data not shown). We also confirmed these results by liver histology. This again supports the idea that the MHC II pathway is not interfering with the development of hepatic fibrosis induced by CCl_4.

Although histological data suggested that there was no difference in the severity of the fibrosis between groups treated by CCl_4, we found a profound increase in the hepatic stellate cells (HSC) staining only in the group of MHCII$^{\Delta/\Delta}$ mice treated by CCl_4. HSC are nonparenchymal cells in the liver and are known to play an important role in fibrosis and tissue repairing [37]. Upon the quiescent HSC activation, they are converted into myofibroblasts, which are responsible for the production of extracellular matrices [38]. The importance of the HSC activation in fibrogenesis was recently reported by Puche et al. [39]. They have elegantly created a transgenic mouse model whose proliferating HSC were selectively killed. By use of the model, Puche et al. demonstrated that these mice had reduced fibrotic area upon CCl_4 treatment compared to their genetically controlled counterparts. This signifies the important role of HSC in the development of hepatic fibrosis.

In our study, despite the hyperactivation of HSC in MHCII$^{\Delta/\Delta}$ mice, the fibrosis formation was strictly comparable between groups. We do not know the exact cause and effect of this HSC induction in MHCII$^{\Delta/\Delta}$ mice treated by CCl_4. We have observed that the expression of the genes of the MHC II pathway was very high in HSC fraction when we separated different cellular types in the liver (hepatocytes, HSC, Kupffer cells, and endothelial cells) (unpublished data). This suggests that the HSC could play an important role for the MHC II reaction. We do not know, however, whether the absence of the pathway in MHCII$^{\Delta/\Delta}$ mice might have affected the HSC proliferation upon CCl_4 stimulation.

On the other hand, the activation of HSC during the development of fibrosis is suggested to be transient [40]. We did not identify when HSC started to be activated in MHCII$^{\Delta/\Delta}$ mice during the CCl_4 treatment. Whether this hyperactivation of HSC has compensated the lack of the MHC II pathway and contributed to the fibrosis formation needs to be further addressed.

The present study clearly demonstrated that the lack of MHC II pathway affected neither NASH induced by high-fat diet nor fibrosis induced by CCl_4 in mice. Despite a large number of publications implying the association between the alleles in the MHC II pathway and drug-induced liver injury and hepatitis C in humans, this study clearly indicated that the MHC II pathway is not required in the development of NASH and fibrosis at least in mice.

Conflict of Interests

G. Willemin, C. Roger, A. Bauduret, and K. Minehira have no conflict of interests.

Authors' Contribution

K. Minehira contributed to the conception and design of the experiments. All *in vivo* experiments and tissue sampling were carried out in the animal facility of the Bugnon 7/9, University of Lausanne, by G. Willemin and K. Minehira. Histological analysis was carried out by C. Roger. The remaining experiments, including any molecular and biochemical analyses, were performed by G. Willemin at the Department of Physiology, University of Lausanne. A. Bauduret contributed to the genotyping strategy. G. Willemin and K. Minehira participated in data analysis and interpretation. K. Minehira wrote the initial draft of the paper, and all authors contributed to its revisions and approved the final version.

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Review Article

Obesity and Inflammation: Epidemiology, Risk Factors, and Markers of Inflammation

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Obesity is a public health problem that has reached epidemic proportions with an increasing worldwide prevalence. The global emergence of obesity increases the risk of developing chronic metabolic disorders. Thus, it is an economic issue that increased the costs of the comorbidities associated. Moreover, in recent years, it has been demonstrated that obesity is associated with chronic systemic inflammation, this status is conditioned by the innate immune system activation in adipose tissue that promotes an increase in the production and release of pro-inflammatory cytokines that contribute to the triggering of the systemic acute-phase response which is characterized by elevation of acute-phase protein levels. On this regard, low-grade chronic inflammation is a characteristic of various chronic diseases such as metabolic syndrome, cardiovascular disease, diabetes, hypertension, non-alcoholic fatty liver disease, and some cancers, among others, which are also characterized by obesity condition. Thus, a growing body of evidence supports the important role that is played by the inflammatory response in obesity condition and the pathogenesis of chronic diseases related.

1. Epidemiology and Obesity

Obesity is actually an epidemic problem in the world; it has become truly a global problem affecting countries rich and poor. An estimated 500 million adults worldwide are obese and 1.5 billion are overweight or obese [1]. Particularly the prevalence of obesity or combined overweight and obesity has increased in Brazil, Canada, Mexico, and United States [2]. Much of the information about obesity among adults rest in the use of body mass index (BMI) to define obesity, which will be defined as a BMI 30 kg/m^2 or greater unless otherwise stated [3]. An examination of national data through 1991 confirmed that significant increases in the United States population had takes place both in adults and children and adolescents [4, 5]. The most recent data from 2005-2006 show that 33.3% of men and 35.3% of women were obese [6]. In Canada, the prevalence of obesity based on measured height and weight has almost doubled in the last two decades and now affects 23% of the adult population [7].

Obesity is a consequence of many risk factors, as increased energy consumption and reduced physical exercise. Many studies also implicate chronic low grade inflammation in the interplay between obesity and metabolic complications, as many chronic degenerative disorders, including atherosclerosis, and are also commonly associated with hypertension, which itself has also been linked recently to inflammation [8, 9]. Obesity and inflammation have been associated with type 2 diabetes, cardiovascular disease, hypertension, stroke, and gallbladder disease, some forms of cancer, osteoarthritis, and psychosocial problems [10]. In obesity subjects, this problem is commonly associated with other metabolic disorders as hyperglycemia and hypertriglyceridemia, which are well-known risk factors for developing chronic liver disease, as nonalcoholic fatty liver disease

(NAFLD) [11, 12]. The prevalence of NAFLD reaches 14% to 21%, but is it as high as 90%–95% in obese persons and up to 70% in diabetic patients [13]. Liver inflammation can be induced by the metabolically active intraabdominal fat, and that the high BMI and large waist circumference are significantly associated with the elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels [14, 15]. Patients with obesity can have elevation of AST and ALT levels, and the reduction of body weight can be achieved with combining diet and physical activity strategies, and reduced levels of aminotransferase [16]. NAFLD and cardiovascular disease have common metabolic risk factors and have 3.7% on mortality; individuals with NAFLD were older, predominantly males, and more likely to be Hispanic. They also had a higher prevalence of all components of metabolic syndrome and cardiovascular disease; however, patients with NAFLD disease did not increase cardiovascular mortality in over 14 years [17].

For example, within the context of chronic HCV and HBV infection, the presence of cirrhosis is the most important risk factor in the development of hepatocellular carcinoma [18]. There are some nonmodifiable risk factors including older age, male gender, and family history, and several modifiable risk factors in hepatocelular carcinoma, of which the most important are alcohol and tobacco [19]. However, identifying additional modifiable risk factors, including diet, is important, including coffee and tea, fructose, iron, red and with meats, types of fat, selenium, and vitamins D and E [20].

Diet and life style play a crucial role in the development of some cancers. Actually in Mexico and others countries, more than one-third of cancer deaths can be avoided through dietary modification. Different mechanisms, including antioxidant, anti-inflammatory, and antiestrogenic processes, have been proposed to explain the protective nature of certain dietary components [21].

2. Obesity and Chronic Inflammation

Inflammation is a physiological response necessary to restore homeostasis altered by diverse stimuli; however, inflammation state chronically established or an excessive response can involve deleterious effects. In overweight and obesity, there exists low-grade chronic inflammation; recent studies have unveiled some of the intracellular pathways of inflammation associated with these conditions; studies in mice and humans evidence that consumption of nutrients may acutely evoke inflammatory responses; so, it is thought that the starting signal of inflammation is overfeeding and the pathway origins in tissues involved in metabolism, that is, adipose tissue, liver, and muscle, which in response of this stimulus triggers the inflammatory response [22, 23]. Compared with lean control, in obese men and women, tissue and liver tissues display an increased activation of kinases such as c-jun N-terminal kinase and the inhibitor of k kinase, which are able to induce the expression of inflammatory cytokines [24, 25]. These kinases regulate downstream transcriptional programs through the transcription factors activator protein-1, nuclear factor κB, and interferon regulatory factor, inducing upregulation of inflammatory mediator gene expression. The increase in cytokines exacerbates receptor activation by establishing a positive feedback loop of inflammation and the inhibitory signaling of metabolic pathways [26].

Likewise, inflammasome and the Toll-like receptors (TLRs) of the innate immune system are activated as well [27, 28]. Now, strong evidences indicate a prominent role of the inflammasome signaling in the development of a chronic proinflammatory state that impairs insulin sensitivity [24].

3. The Inflammasome

Inflammasome is a macromolecular innate immune cell sensor that initiates the inflammatory response. Recognition of diverse noxious signals by the inflammasome results in activation of caspase-1, which subsequently induces secretion of potent proinflammatory cytokines, particularly interleukin-1β (IL-1β). In this way, inflammasome-mediated processes are important in regulating metabolic processes [24, 29].

The inflammasome is a heptamer formed by monomers containing Nod-like receptors (NLRs), the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain), and the enzyme caspase-1. NLRs are characterized by a structure composed of a central domain that mediates nucleotide-binding and oligomerization (NOD or NBS domain), a C-terminal leucine-rich domain (LRR), and a variable N-terminal region required for protein-protein interactions. When assembled as inflammasome, NLR activates caspase-1, which converts pro-IL-1β into active IL-1β [30, 31].

In the human being, the NLR family consists of 22 members, classified in 4 subfamilies, NLRA, NLRB, NLRC, and NLRP, on basis of their N-terminal domain configuration. They interact with the inflammasome-associated proteins ASC and caspase-1 [32].

A member of the NLRP, named NLRP3, has been linked to metabolic stress, insulin resistance, and type 2 diabetes. NLRP3 inflammasome activation in obesity promotes macrophage-mediated T cell activation in adipose tissue and impairs insulin sensitivity creating a chronic proinflammatory state that impairs insulin sensitivity. Inflammasome activation can be induced by hyperglycemia, reactive oxygen species, palmitate, lipopolysaccharides, and uric acid, among other substances [24]. These findings highlight the potential molecular intervention in pathways regulating caspase-1 activation for management of chronic inflammation [29–31, 33].

Recent studies show that a protein upregulated by glucose, the thioredoxin interacting protein (TXNIP), interacts with NLRP3, leading to IL-1β secretion and hampering of pancreatic β-cell function [34, 35].

4. Inflammatory Cytokines

The origin of inflammation during obesity and the underlying molecular mechanisms that explain its occurrence are not yet fully understood, but pro-inflammatory cytokines play a central role. In obesity, there are higher circulating concentrations of inflammatory cytokines than in lean beings,

and it is believed that they play a role in causing insulin resistance. The main source of pro-inflammatory cytokines in obesity is the adipose tissue; they are mainly produced by infiltrating macrophages, although adipocytes play a role. In this way, blood concentrations of these cytokines are lowered following weight loss [22, 23]. The main cytokines responsible of chronic inflammation are tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), and the inflammasome-activated IL-1β mentioned earlier.

TNF-α is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism, with numerous effects in adipose tissue, including lipid metabolism and insulin signaling. Circulating TNF-α is increased in obesity and decreased with weight loss. TNF-α promotes the secretion of other powerful pro-inflammatory cytokine, IL-6, and reduces anti-inflammatory cytokines like adiponectin. TNF-α induces adipocytes apoptosis and promotes insulin resistance by the inhibition of the insulin receptor substrate 1 signaling pathway [36, 37].

IL-6 is a cytokine that plays important roles in acute phase reactions, inflammation, hematopoiesis, bone metabolism, and cancer progression. IL-6 regulates energy homeostasis and inflammation; it is capable of suppressing lipoprotein lipase activity, and it controls appetite and energy intake at hypothalamic level [38]. IL-6 is important in the transition from acute inflammation to chronic inflammatory disease. It contributes to chronic inflammation in conditions such as obesity, insulin resistance, inflammatory bowel disease, inflammatory arthritis, and sepsis when deregulated [39].

IL-1β is a pyrogenic cytokine. It is mainly produced by blood monocytes in response to infection, injury, or immunologic challenge; it causes fever, hypotension, and production of additional pro-inflammatory cytokines, such as IL-6. IL-1β is formed from its pro-IL-1β inactive precursor by the inflammasome. In this way, IL-1β has now emerged as a prominent instigator of the pro-inflammatory response in obesity [24].

Important advances have been reached in the last decade in the understanding the role of cytokines and the inflammasome in obesity, chronic inflammation, insulin resistance, and type 2 diabetes. However, further research is required to better understand the underlying mechanisms as they are potential intervention points in the search of new therapeutically modalities for these global health problems.

5. Markers of Inflammation

Several chronic diseases involve an inflammatory response characterized by the increase of cytokines and serum concentrations of acute-phase reactants (markers of active inflammation) such as fibrinogen, C-reactive protein (CRP), complement, serum amyloid A, haptoglobin, sialic acid and low albumin concentrations [40]. Acute-phase reactants are synthesized in the liver, and its production is regulated by cytokines, including IL-6 and TNF-alpha [41–44]. The CRP, consideredes the classic sensitive acute-phase reactant, is a very sensitive systemic marker of inflammation, and its serum concentration increases rapidly in response to a variety of stimuli. This protein is present in low concentrations under normal conditions [45, 46].

Visceral adipose tissue may produce inflammatory mediators, which induce the production of acute-phase reactants in hepatocytes and endothelial cells [47]. In fact, because it has been shown that adipocytes express and secrete TNF-alpha, adipose body mass may be an important mediator to explain the relation between obesity and inflammation [48]. Some studies have shown that abdominal adiposity is associated with elevation of CRP levels, independent of body mass index (BMI), which is a measure of general adiposity. The proportion of people with elevated hs-CRP was significantly higher in those individuals with abdominal adiposity than control subjects, although they had a similar BMI [49]. IL-6 is a pro-inflammatory cytokine synthesized by adipose tissue, endothelial cells, macrophages, and lymphocytes. The CRP is synthesized in the liver largely in response to IL-6 stimuli [50]. Individuals with obesity are at increased risk for various chronic diseases, several of which are also characterized by elevated CRP concentrations. Because adipose tissue is a major source of pro-inflammatory cytokines such as IL-6 and TNF-alpha, both cytokines increase hepatic lipogenesis [51, 52] and trigger a systemic acute-phase response [41].

In recent years, it has been demonstrated that obesity is associated with low-grade inflammatory process characterized by the increase in circulating levels of pro-inflammatory cytokines such as IL-6, TNF-alpha, and acute-phase proteins (CRP and haptoglobin) in healthy obese subjects [53–56]. This phenomenon is also observed in obese children who have higher CRP levels than normal weight children [57]. Some studies have reported that weight loss, through diet, is associated with reduction in circulating levels of IL-6, TNF-alpha, CRP, and other markers of inflammation, independently of age, sex, and BMI [58, 59]. Similarly, weight reduction observed in subjects after gastric bypass shows decrease of CRP and IL-6 levels [60].

6. Metabolic Syndrome

The metabolic syndrome is characterized as the presence of three or more of the following features: obesity, hyperglycemia, hypertension, low HDL cholesterol levels, and/or hypertriglyceridemia [61–64]. Although pathogenic mechanisms are poorly understood, a central role has been attributed to the pro-inflammatory cytokines TNF-alpha [65] and IL-6 [66], since both are synthesized by adipose tissue. This syndrome has been associated with markers of inflammatory activity, such as CRP [67–75], IL-6 [76, 77], serum amyloid A [78, 79], and soluble adhesion molecules [73, 75, 80, 81].

Risk Factors. Low-grade chronic inflammation is associated with metabolic syndrome [82] and some features of insulin resistance [83]. Other studies have demonstrated significant correlation between CRP levels with features of the metabolic syndrome, including adiposity, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and low HDL cholesterol [84, 85]. Few studies have reported the association between

CRP and development of metabolic syndrome [50, 86]. In addition, it has been observed that elevated hs-CRP levels are associated with increased risk for incident cardiovascular events among individuals as having the metabolic syndrome [87]. Inflammation has been proposed as common part of different metabolic disturbances of insulin, glucose, and lipids that influence the underlying development of metabolic syndrome [50].

Also, it has shown that CRP adds independent prognostic information on severity of metabolic syndrome [87]. Given the evidence, it has been proposed that CRP is an additional component of metabolic syndrome [88]. In one study, it was reported that elevated levels of CRP (≥3 mg/L) may increase the risk of metabolic syndrome mediated through obesity and factors related to insulin resistance [50].

Treatment. Observational studies have shown that dietary patterns similar to the Mediterranean diet, rich in fruit and vegetables and high in monounsaturated fats and fiber, resulted in decrease prevalence of the metabolic syndrome [89–91]. In addition, interventional studies also demonstrated a decrease in markers of inflammation in subjects with metabolic syndrome consuming Mediterranean diet and/or national dietary guidelines [92, 93].

Studies that evaluate markers of inflammation in individuals with metabolic syndrome are scarce; however, some have shown anti-inflammatory effects of statin therapy [94, 95]. Because subjects with metabolic syndrome exhibit increased inflammation, after therapeutic lifestyle changes, statins could be a therapeutic option.

7. Cardiovascular Disease

In the last years, different markers of inflammation (such as CRP, IL-6, and TNF-alpha, among others) have been studied in prediction of coronary events; on this regard, CRP is the most important marker for cardiovascular disease [96].

Risk Factors. Circulating elevated levels of inflammatory markers, such as CRP, TNF-alpha, and IL-6, are associated with increased risk of developing cardiovascular disease [97–102]; even some acute-phase reactants may also contribute to their pathogenesis [103]. Though in mild degree, chronic elevation of CRP levels, even within normal value range, is an independent predictor of future cardiovascular events [99, 104]. Stratified ranges of high-sensitivity CRP levels of <1, 1–3, and >3 mg/L correspond to low, moderate, and high risks for future cardiovascular events. Previously, some studies have found a significative association between CRP and cardiovascular risk [105, 106]. This finding was observed for the first time over 50 years ago, where increased CRP level, after myocardial infarction, was identified as marker of poor prognostic [87]. Later, the European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group reported that CRP concentrations were higher in the patients who had coronary events than in those without such events [107]. In addition, the Cholesterol and Recurrent Events Trial showed that elevated CRP levels are associated with major risk of coronary events after myocardial infarction [108]. A growing body of evidence has corroborated that inflammation is a strong predictor of future cardiovascular events [96–99, 104, 109–114].

Furthermore, hsCRP is better marker of cardiovascular disease than others acute-phase reactants, cytokines, and soluble adhesion molecules [115]. Thus, supported by a large number of observational studies and meta-analyses, CRP is considered as a mediator of cardiovascular disease [116], independently of age, smoking, cholesterol levels, blood pressure, and diabetes among others traditional risk factors evaluated in the clinical setting [117]. Thus, CRP is one of the most well-documented emerging cardiovascular disease risk factors [118, 119].

Treatment. Some interventional studies using Mediterranean diet and others characterized by increased intake of mustard or soybean oil, fruits, vegetables, nuts, and whole grains reduced the rate of cardiovascular disease with significant anti-inflammatory effect [120, 121]. Also, various observational and interventional studies found that intake of omega-3 and omega-6 fatty acids and alpha-linolenic acid resulted in lower risk of cardiovascular disease and lower concentrations of markers of inflammation [122–128]. Moreover, several studies have shown that statin therapy is associated with reduced inflammation and cardiovascular risk reduction [108, 129–141].

8. Diabetes

Several studies have shown that subclinical systemic inflammation, as measured by elevated levels of CRP and IL-6, predicts the development of diabetes [142–149]. In fact, IL-6 may interfere with insulin signalling through induction of proteins that bind to the insulin receptor [150]. On this regard, a growing body of evidence supports the hypothesis that chronic systemic inflammation contributes to decrease of insulin sensitivity at peripheral tissues [40, 45, 151, 152].

Risk Factors. Several studies in healthy subjects have confirmed that elevated levels of CRP and cytokines IL-6 and TNF-alpha are associated with insulin resistance [84, 85, 153–155]. In addition, it has been shown that in the individuals with impaired glucose tolerance [156, 157], the low-grade chronic inflammation is related to glucose metabolic disturbances.

It has been reported that TNF-alpha is overexpressed in the adipose and muscle tissues of obese and insulin-resistant nondiabetic subjects, overexpression that is positively correlated with insulin resistance [48, 158–160]. Interestingly, circulating TNF-alpha levels are higher in type 2 diabetes [161–163] as compared with IFG/IGT [156]. In addition, several cross-sectional studies have shown an increase of CRP levels in patients with diabetes [142, 143, 164] and the increase of CRP, IL-6, and TNF-alpha in subjects with IGT [40, 165].

Moreover, in obesity there are elevated levels of several kinases such as protein kinase C isoforms, I Kappa B Kinase-β, and c-jun-terminal kinase, and these kinases have been implicated in alteration of insulin signaling by promoting serine phosphorylation of insulin receptor substrate which

is associated with suppression of tyrosine phosphorylation of this substrate [166]. Also, various studies have demonstrated that nutrient excess and obesity are associated with elevated levels of free fatty acids, which can induce both insulin resistance in peripheral tissues and activation of innate immunity [28, 167–172].

Furthermore, it is difficult to set cut-point values to predict risk of development disease because intermediate values of CRP are at moderate risk for metabolic disturbances. However, it has been reported that patients with diabetes and CRP values >3 mg/L have 51% higher risk of all-cause mortality and 44% higher risk of cardiovascular mortality than subjects with diabetes and CRP <3 mg/L of similar age and sex, independently of classical risk factors such as lipids, blood pressure, and glycemia [173].

Treatment. In clinical field, there are different therapeutic options, such as genetic, biochemical, and pharmacological targeting of inflammatory signalling pathways improving insulin action, a central problem in the pathophysiology of type 2 diabetes [174]. Existing evidence about inhibiting specific inflammatory kinases pathway improves insulin action in animal models [175, 176]. Pharmacological therapeutics using thiazolidinediones exhibited anti-inflammatory effects inhibiting both adipocyte and macrophage function in obesity and type 2 diabetes [177]. Various clinical studies, using anti-inflammatory drugs to treat type 2 diabetes and even prediabetes, showed improvements in beta-cell function and insulin sensitivity, reducing glucose levels [34, 178–182]. In addition, others studies in patients with type 2 diabetes taking statins have demonstrated a beneficial and additive effect on markers of inflammation [183–186], which could be an alternative therapeutic for this disease; however, the clinical practice recommendations should be considered about the appropriate use of statin therapy because basic studies have documented controversial results regarding the beneficial and adverse effects on insulin secretion and sensitivity [187].

9. Conclusion

The origin of inflammation during obesity and the underlying molecular mechanisms that explain its occurrence are not still fully understood, but pro-inflammatory cytokines play a central role. In obesity, there are higher circulating concentrations of inflammatory cytokines than in lean beings, and it is believed that they play a role in causing insulin resistance. The main source of proinflammatory cytokines in obesity is the adipose tissue; they are mainly produced by infiltrating macrophages, although adipocytes play a role. Obesity is a consequence of many risk factors, as increased energy consumption and reduced physical exercise. Many problems ecist in patients with obesity, as cardiovascular disease, diabetes, metabolic syndrome, and NAFLD, among others, predicting the risk of future cardiovascular events and mortality. Different mechanisms, including antioxidant, anti-inflammatory, fiber diet, and antiestrogenic processes, have been proposed to explain the protective nature of certain dietary components, particularly, components of Mediterranean diet which could be an important therapeutic lifestyle change which allows to avoid the development of metabolic diseases.

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Research Article

Independent Association between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease: A Systematic Review and Meta-Analysis

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Nonalcoholic fatty liver disease (NAFLD) is closely correlated with insulin resistance and several metabolic syndrome features, but whether it could increase the risk of cardiovascular disease remains undefined. To assess the association between NAFLD and the risk of cardiovascular outcomes, we systematically searched the MEDLINE, Embase, and the Cochrane Library database (1947 to October 2012) by using Medical Subject Heading search terms and a standardized protocol. Randomized controlled trials, case-control, and prospective studies carried out in human adults, in which the unadjusted and multivariate adjusted odds ratios with corresponding 95% confidence interval (CI) for cardiovascular disease with NAFLD were reported. The search yielded 4 cross-sectional studies and 2 prospective cohort studies including 7,042 participants. The pooled effects estimate showed that NAFLD was a predictor of cardiovascular disease (odds ratio 1.88, 95% CI, 1.68 to 2.01; $P < 0.001$). The random effects summary estimate indicated that NAFLD retained a significant association with cardiovascular outcomes independent of conventional risk factors after adjustment for established cardiovascular risk factors (odds ratio 1.50, 95% CI, 1.21 to 1.87; $P < 0.001$). These results indicate that NAFLD is a strong independent predictor of cardiovascular disease and may play a central role in the cardiovascular risk of metabolic syndrome.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a wide spectrum of hepatic disorders in clinical practice [1], the prevalence in the general population is 10% ~30%, and the number increases greatly whether in developing or developed countries [2–5]. It has been convincingly associated with insulin resistance and metabolic syndrome (MS); most patients are overweight or frankly obese, with altered glucose metabolism, dyslipidemia, and raised blood pressure, all contributing to the disorders [6–8].

However, the clinical and public health significance of NAFLD is not well established. People with NAFLD harbor the same cardiovascular risk factors (hypertension, dyslipidaemia, obesity, physical inactivity, insulin resistance, endothelial dysfunction, and inflammation) that place them at high risk of cardiovascular events [9, 10]. Recently, some studies showed that subjects with NAFLD have an elevated risk of increased carotid intima media thickness [11, 12], reduced endothelial function [13], increased coronary artery calcification [14, 15], and increased arterial stiffness [16]. However, other studies indicated that NAFLD was not associated with MS and cardiovascular disease [17]. Despite these results, it remained controversial whether NAFLD was a marker or an independent mediator that promotes cardiovascular disease, and the effect of NAFLD on the risk of future cardiovascular events has not been well established.

Hence, we performed a systematic review and meta-analysis with the most updated prospective data to evaluate

the association of NAFLD with the risk of incident cardiovascular outcomes in patients.

2. Methods

The search strategy was in accordance with the recommendations of the meta-analysis of observational studies in epidemiology (MOOSE) group. We searched EMBASE (1947 to October 16, 2012), MEDLINE (1947 to October 16, 2012), The Cochrane Library (1947 to October 16, 2012), Science Citation Index (Web of Knowledge) (1947 to October 16, 2012), and PubMed (1947 to October 16, 2012), using the search terms "nonalcoholic fatty liver disease" or "NAFLD" or "fatty liver" AND "cardiovascular disease" or "myocardial ischemia" or "MI" or "myocardial infarct" or "ischemic heart disease" or "coronary heart disease" or "coronary artery disease" or "angina" or "stroke" or "cerebrovascular disease" or "cerebrovascular attack" or "cerebral ischemia" or "brain ischemia" or "intracranial hemorrhage." The search had no language restriction.

2.1. Inclusion and Exclusion Criteria. An article was considered relevant if it reported quantitative estimates of the unadjusted and (or) multivariable adjusted (i.e., age, sex, smoking history, diabetes duration, HbA1c, LDL cholesterol, GGT (gamma-glutamyl transpeptidase) levels, and use of medications (i.e., hypoglycemic, antihypertensive, lipid-lowering, or antiplatelet drugs), or additional adjustment for the presence of metabolic syndrome and/or NAFLD) odds ratio with corresponding 95% confidence interval (CI) for the log relative risk for cardiovascular events. NAFLD patients were evaluated at least by abdominal ultrasound or computed tomography (CT). Cardiovascular events include coronary heart disease (such as myocardial infarction, angina pectoris, and ischemic stroke), cerebrovascular disease (such as cerebral hemorrhage), and peripheral vascular disease. The criteria of National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) are used to characterize the metabolic syndrome (MS) [18]. Unpublished papers, nonhuman studies, letters/case reports, studies enrolling <10 subjects or subjects aged <12 years, editorials, reviews, no cardiovascular endpoints, no multivariate adjusted cardiovascular events estimate, or using inadequate case definition were excluded.

2.2. Data Extraction and Quality Assessment. The data extraction was performed independently by two authors and included first author, date of publication, location of research group, number of analyses, characteristics of participants, study design, outcome measures, and selection criteria. We extracted the unadjusted and (or) multivariable adjusted odds ratio for cardiovascular events and corresponding 95% CI in the statistical analysis. Reference lists of the retrieved articles were searched for additional publications. The quality of the selected studies was assessed independently by two authors using the Newcastle-Ottawa Scale (NOS) for cohort and cross-sectional studies. The NOS used a "star" rating system to judge quality based on three aspects of the study: selection of study groups, comparability of study groups, and ascertainment of either the exposure or outcome of interest [19]. Any discrepancies were addressed by a joint reevaluation of the original article with another author.

2.3. Statistical Analysis. The results of each study were reported as an odds ratio. To measure the outcome, the DerSimonian-Laird method and random-effects model (REM) were used. Results were expressed as pooled odds ratios (OR (95% confidence intervals, CIs)). We assessed whether a significant level of difference existed using Mantel-Haenszel chi-square tests. If the chi square test was significant below $P = 0.05$, we quantified the amount of heterogeneity using I^2 statistics. We considered I^2 above 50% as indicative of substantial heterogeneity [20]. The potential for publication bias was addressed by drawing funnel plots and visual assessment [21]. Meta-analyses were performed for unadjusted and multivariate adjusted cardiovascular events estimate with NAFLD and MS, separately. We used the Stata10 software package for the meta-analysis of observational studies. In the forest plots, OR values > 1 represent a direct association and <1 an inverse association. The size of the squares was correlated with the weight of the respective study [21].

3. Results

3.1. Search Results. The literature search yielded a total of 802 potentially relevant abstracts. Seven hundred and forty articles were excluded by review of abstract because they did not address people with NAFLD, or they did not assess the association between NAFLD and cardiovascular disease. By reviewing full articles, 56 articles were rejected because they (1) have no multivariate adjusted cardiovascular events estimate, (2) fatal endpoints only, (3) had no original data (review, editorials), or (4) were duplicate publications. Finally, six studies (Figure 1) were included in our analysis [8, 22–26].

3.2. Characteristics of the Studies. The main characteristics of the studies included in this analysis are provided in Table 1. Among them, one study originated from Korea, one from Israel, three from Italy (they were conducted by the same research group among diabetics, but three studies did not include the same participants), and one from Japan, a total of 7,042 participants. According to the NOS score, the six studies were of high quality.

3.3. Outcome Results. These six studies all reported adjusted odds ratio with corresponding 95% CI, which allowed us to pool their data into a further analysis. Figure 2 shows a univariate meta-analysis unadjusted odds ratio [24] for cardiovascular disease with NAFLD. The analyses were based on the fixed-effect model. Heterogeneity chi-square = 8.67, d.f. = 4, and $P = 0.07$. We did not find significant evidence of heterogeneity across studies (P for heterogeneity > 0.05). The fixed effects summary estimate shows an increased risk of cardiovascular disease (odds ratio 1.88, 95% confidence

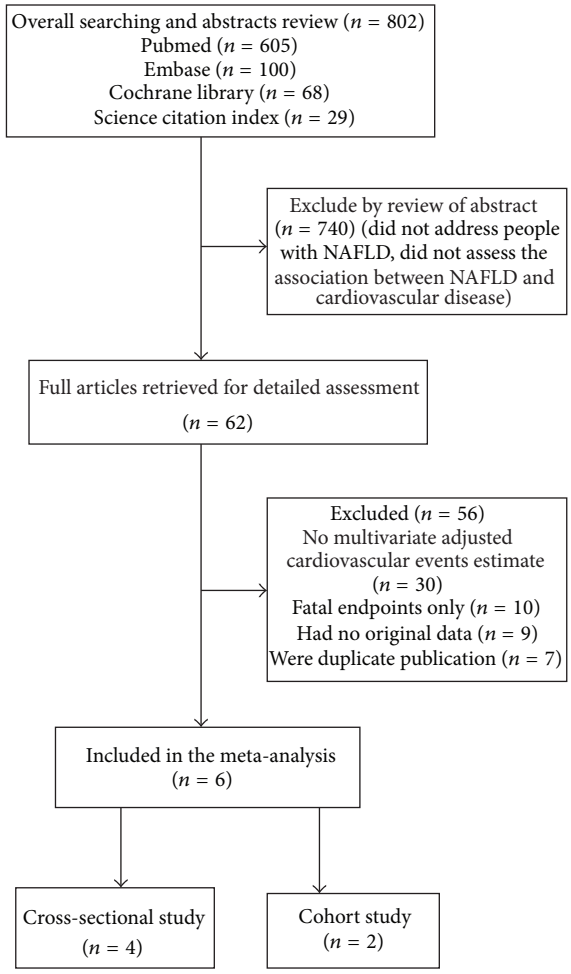

Figure 1: Flow diagram of studies assessed and included.

interval 1.68 to 2.10; $P < 0.001$) and no major asymmetrical appearance in the funnel plot.

Thus, we did a multivariable adjusted odds ratio meta-analysis based on the primary outcome reported in all studies for cardiovascular disease with NAFLD. The analyses were based on the random-effect model and are presented in Figure 3. Heterogeneity chi-square = 11.61 (d.f. = 5), $P = 0.04$, I-squared = 56.9%, and $\tau 2 = 0.0373$. The random effects summary estimate shows an increased risk of cardiovascular disease after adjustment for established cardiovascular risk factors (age, sex, diabetes duration, HbA1c, smoking history, LDL cholesterol, GGT levels and use of medications (i.e., hypoglycemic, antihypertensive, lipid-lowering or antiplatelet drugs) and NCEP ATP III-defined MS) (odds ratio 1.50, 95% CI 1.21 to 1.87; $P < 0.001$). We found evidence of heterogeneity across studies (P for heterogeneity < 0.05) but no major asymmetrical appearance in the funnel plot.

We also did meta-analysis stratifying by study type for cross-sectional and prospective studies based on the primary outcome reported in five studies. The heterogeneity between cross-sectional studies chi-square = 1.82, df = 2, $P = 0.403$, and I-squared = 0.0%. We did not find significant evidence of heterogeneity across cross-sectional studies (P for heterogeneity > 0.05). The subgroup summary estimate shows increased risk of cardiovascular disease (odds ratio 1.82, 95% CI 1.60 to 2.07; $P < 0.001$) and no major asymmetrical appearance in the funnel plot. The heterogeneity between the prospective studies chi-square = 5.97, df = 1, $P = 0.015$, and I-squared = 83.3%. We found significant evidence of heterogeneity across prospective studies ($P < 0.05$). The subgroup summary estimate shows increased risk of cardiovascular disease (odds ratio 2.05, 95% CI 1.65 to 2.55; $P < 0.001$) and no major asymmetrical appearance in the funnel plot. We also did meta-analysis based on half of the studies only included diabetic patients. The heterogeneity chi-square = 4.98, df = 2, $P = 0.083$, and I-squared = 59.8%. We did not find significant evidence of heterogeneity across studies ($P > 0.05$). The estimate shows an increased risk of cardiovascular disease (odds ratio 1.34, 95% CI 1.17 to 1.54; $P < 0.001$) and no major asymmetrical appearance in the funnel plot.

4. Discussion

NAFLD is a hepatic manifestation of the metabolic syndrome, it is closely related to other clinical features of the metabolic syndrome, and thus cardiovascular disease is increased in NAFLD and represents the main cause of death in these patients. However, given the shared features between NAFLD, the metabolic syndrome, and traditional cardiovascular risk factors, it remains uncertain whether NAFLD is an independent risk factor for increased cardiovascular event [22, 25, 26]. Several previous studies have demonstrated that patients with NAFLD have significantly higher rates of prevalent coronary, cerebrovascular, and peripheral vascular disease than their counterparts without NAFLD [9–11, 27–31]. However, the lack of diagnostic uniformity and difficulty in accurately quantifying the severity of NAFLD in the various published studies make interpretation of the results challenging and sometimes contradictory [32, 33]. There is an urgent need to ensure a more homogeneous evaluation of study outcomes [34, 35]. To provide a more objective basis for clinical recommendations, we conducted a meta-analysis, which recruited a total of 7,042 individuals from 2 prospective and 4 cross-sectional studies. To our knowledge, this is the first meta-analysis on this topic that provides a complete analysis of the potentially harmful role of NAFLD on cardiovascular disease. In our previous cross-sectional study which was conducted among 560 cases of in-patients type 2 diabetes mellitus patients from January 2002 to January 2009 in Southern China, we found that NAFLD was associated with a higher prevalence of coronary heart disease in type 2 diabetes, and that plasma ALT levels may act as a marker [29]. In this meta-analysis, we confirmed previous data demonstrating the high prevalence of cardiovascular disease in NAFLD patients [14]. More importantly, our findings extend the work of recent small studies showing that patients with NAFLD, as assessed by ultrasonography or computed tomography (CT), had a significant association with cardiovascular mortality.

Table 1: Characteristics of studies include in meta-analysis.

First author (year)	Country	Participants		Study design	Followup (person-year)	NOS score	Outcome measures
		No. in analysis	Characteristics				
Assy (2010)	Israel	800	Individuals with law to intermediate risk for CAD and presence of fatty liver	Cross-sectional	NA	7	Coronary heart disease coronary atherosclerosis coronary plague (%)
Choi (2008)	Korea	659	Healthy people	Cross-sectional	NA	7	Carotid atherosclerosis
Targher (2007)	Italy	2392	Diabetic patients	Cross-sectional	NA	7	Coronary heart disease cerebrovascular disease peripheral vascular disease
Hamaguchi (2012)	Japan	1647	Healthy people	Cohort	7115	8	Coronary heart disease ischemic stroke cerebral hemorrhage
Targher (2006)	Italy	800	Diabetic patients	Cross-sectional	NA	7	Coronary disease peripheral disease cerebral disease
Targher (2005)	Italy	744	Diabetic patients	Cohort	3720	7	Myocardial infarction coronary artery bypass grafting ischemic stroke cardiovascular events death

NOS score = Newcastle-Ottawa scale: used for quality assessment. We assigned NOS scores of 1–3, 4–6, and 7–9 for low, intermediate, and high-quality studies, respectively. These six studies are all adjusted for confounders, such as age, sex, diabetes duration, HbA1c, smoking history, LDL cholesterol, GGT levels and use of medications (i.e., hypoglycemic, antihypertensive, lipid-lowering, or anti-platelet drugs), and NCEP ATP III-defined MS.

In our analysis, we found that NAFLD was a predictor of cardiovascular events (pooled univariate odds ratio 1.88, 95% CI 1.68 to 2.10; $P < 0.001$). Even after adjustment for confounders (age, sex, diabetes duration, HbA1c, smoking history, LDL cholesterol, GGT levels and use of medications (i.e., hypoglycemic, antihypertensive, lipid-lowering or antiplatelet drugs), and NCEP ATP III-defined MS), the association remained significant (pooled multivariate odds ratio 1.50, 95% CI 1.21 to 1.87; $P < 0.001$). This analysis shows that NAFLD is an independent novel predictor for cardiovascular events, even when other components of the metabolic syndrome are taken into account. Because of the link between the two disorders, and that the majority of patients diagnosed with NAFLD are asymptomatic [36], more careful surveillance of these patients will be needed [37]. Healthcare providers should recognize this higher risk of cardiovascular disease. Patients should be educated as it is our experience that they become singularly focused on liver enzymes and ignore more important cardiovascular health [35, 38, 39]. All NAFLD patients should be evaluated for their metabolic, cardiovascular, and liver-related risk.

5. Strengths and Limitations of Study

Our study has several limitations. Firstly, similar to other reports [9, 16, 33, 40], the diagnosis of NAFLD obtained in our study was based on ultrasonography or computed tomography (CT) and the exclusion of known causes of chronic liver disease but was not confirmed by liver biopsy. Although liver biopsy remains the gold standard for NAFLD diagnosis and evaluation, it is difficult to conduct in large populations, and ultrasonography remains the most common way of diagnosing NAFLD in clinical practice due to its good sensitivity and specificity in detecting moderate and severe steatosis [41]. Secondly, NAFLD ranges from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH) [1, 16, 30, 37]. One recent meta-analysis showed that compared to SS, NASH has a higher liver-related (OR for NASH: 5.71, 2.31–14.13; OR for NASH with advanced fibrosis: 10.06, 4.35–23.25) but not cardiovascular mortality (OR: 0.91, 0.42–1.98) [30]. In our study, we did not take the NAFLD histological subtypes into account.

Despite these limitations, our study also has notable strengths. First, this analysis was obtained by pooling data

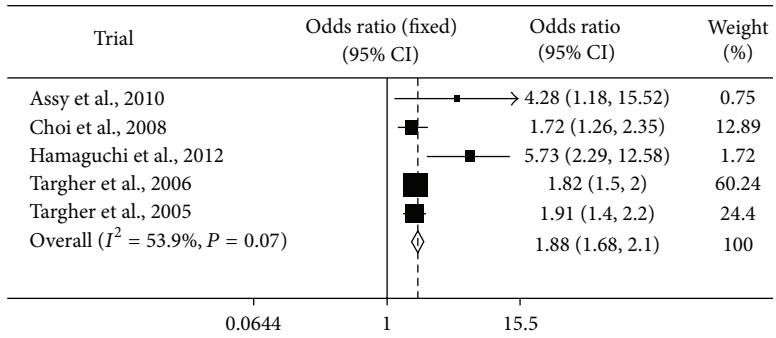

Figure 2: Summary estimates for Mantel-Haenszel odds ratios, the corresponding 95% CI limits, and significance (P value) were estimated by fixed effects metaregression analysis for cardiovascular disease between the two groups (NAFLD patients and controls). In the graph, numbers indicate OR values, filled squares stand for the effect of individual studies, and the diamond expresses combined fixed effects.

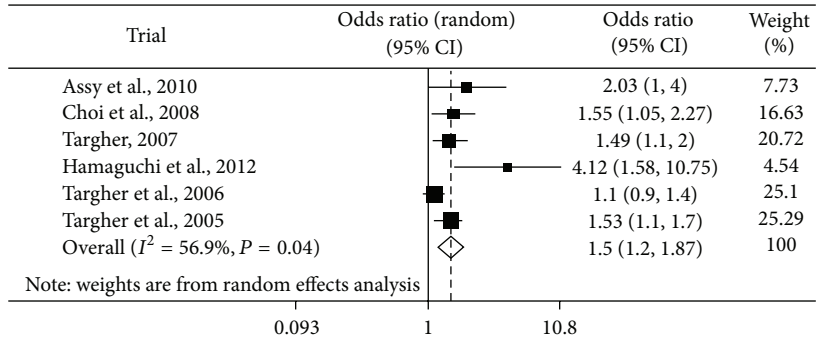

Figure 3: Summary estimates (after adjustment for confounders) for Mantel-Haenszel odds ratios, the corresponding 95% CI limits, and significance (P value) were estimated by random effects metaregression analysis for cardiovascular disease with NAFLD patients.

from a number of clinical trials; the heterogeneity between studies was less evident, which significantly increased the statistical power of the analysis compared to a single study. Second, the quality of studies included in the current meta-analysis was based on the NOS. All of them were of high quality. Third, the included studies originated from different countries and included a variety of ethnic backgrounds, allowing for the generalization of our results. Finally, because this meta-analysis was based on unadjusted and multivariate adjusted estimates, separately, the results of it are possibly the most precise estimate available of the strength of the relation between NAFLD and future risk of cardiovascular events.

In conclusion, our results suggest that NAFLD is a strong independent predictor of cardiovascular disease and may play a central role in the cardiovascular risk of MS. When NAFLD is diagnosed, the person's overall cardiovascular risk factor profile should be reviewed to ensure that risk factors are being appropriately modified.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Hepatic Steatosis and Thyroid Function Tests in Overweight and Obese Children

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Objectives. Associations between thyroid function and nonalcoholic fatty liver disease (NAFLD) are unknown in childhood. Thus, the aim of the present study was to investigate in 402 consecutive overweight/obese children the association between thyroid function tests and hepatic steatosis as well as metabolic variables. *Methods*. Hepatic steatosis was diagnosed by ultrasound after exclusion of infectious and metabolic disorders. Fasting serum samples were taken for determination of thyroid function (TSH, FT4, and FT3), along with alanine aminotransferase (ALT), lipid profile, glucose, insulin, and insulin resistance (IR). *Results*. Eighty-eight children (21.9%) had TSH above the normal range (>4.0 mIU/L). FT3 and FT4 were within the reference intervals in all subjects. Elevated TSH was associated with increased odds of having hepatic steatosis (OR 2.10 (95% CI, 1.22–3.60)), hepatic steatosis with elevated ALT (2.42 (95% CI, 1.29–4.51)), hypertriglyceridemia, elevated total cholesterol, and IR as well as metabolic syndrome (considered as a single clinical entity), after adjustment for age, gender, pubertal status, and body mass index-SD score (or waist circumference). *Conclusions*. In overweight/obese children, elevated TSH concentration is a significant predictor of hepatic steatosis and lipid and glucose dysmetabolism, independently of the degree of total and visceral obesity.

1. Introduction

Over the last two decades, the rise in the prevalence rates of overweight and obesity may explain the emergence of nonalcoholic fatty liver disease (NAFLD) as the leading cause of liver disease worldwide [1]. NAFLD, a novel component of metabolic syndrome (MetS) [2], is a spectrum of fat-associated liver conditions that can result in end-stage liver disease and the need for liver transplantation [1, 3]. Simple steatosis, or fatty liver, occurs early in NAFLD and may progress to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis with increased risk of hepatocellular carcinoma [1, 3].

Several previous studies have addressed the association between thyroid function and NAFLD [4–10]. The results of these studies support an association between overt or subclinical thyroid disease and NAFLD. This seems to be biologically plausible given the common clinical and biochemical features of central obesity, insulin resistance (IR), hypertension, hypertriglyceridemia, and lipid peroxidation observed in both NAFLD and hypothyroidism [5, 11]. However, confirmation and further characterization of clinical data supporting this association are needed. To our knowledge, none of the previous studies have investigated the relationship between hepatic steatosis and thyroid function in childhood. Because small abnormalities in thyroid function are common, thyroid function may importantly influence the prevalence of NAFLD in a population of overweight/obese children and adolescents. We therefore tested the hypothesis that even slightly elevated serum levels of TSH indicating mild thyroid failure might be associated with an increased risk for NAFLD. To this end we explored in overweight/obese children with no clinical, autoantibody, and ultrasonographic evidence of thyroid disease the association between thyroid function tests (thyroid-stimulating hormone (TSH), and free thyroxine (FT4), free triiodothyronine (FT3)) and hepatic steatosis as well as MetS and its parameters.

2. Materials and Methods

2.1. Study Subjects. This is a cross-sectional study carried out at the Hepatology outpatient Clinic of the Department of Pediatrics, Sapienza University of Rome, Italy, between April 2010 and September 2012. The children included were all those being overweight (body mass index (BMI) higher than the age- and sex-specific 85th percentile) or obese (BMI higher than the age- and sex-specific 95th percentile) [12] aged 6–16 years. However, the following subjects were excluded: (a) those with renal disease; type 1 or 2 diabetes; any condition known to influence body composition, insulin action, or insulin secretion (e.g., glucocorticoid therapy, overt hypothyroidism, and Cushing's disease); (b) those with known thyroid disease, current or past history of thyroid hormone or antithyroid drug intake, thyroid alterations in volume and morphology at ultrasound, and positive antithyroglobulin (anti-TG) and anti-thyroperoxidase (anti-TPO) antibodies; (c) those with any laboratory or clinical evidence suggesting an alternate or coexistent underlying chronic liver disease including hepatic virus infections (Hepatitis A–E and G, cytomegalovirus, and Epstein-Barr virus), autoimmune hepatitis, metabolic hepatic disease, α-1-antitrypsin deficiency, cystic fibrosis, Wilson's disease, hemochromatosis, and celiac disease; and (d) those with history of alcohol consumption and smoking (where appropriate).

All study participants underwent physical examination including measurements of weight, standing height, BMI, waist circumference (WC), determination of the stage of puberty, degree of obesity, and systolic blood pressure (BP) and diastolic BP. The pubertal stage was categorized into two groups (prepubertal: boys with pubic hair and gonadal stage I, and girls with pubic hair and breast stage I; pubertal: boys with pubic hair and gonadal stage ≥II and girls with pubic hair stage and breast stage ≥II). WC was obtained at the midpoint between the lowest rib and the iliac crest. The measurement was made at the end of a normal expiration while the subjects were in a standing position. The degree of obesity was quantified using Cole's least mean-square method, which normalizes the skewed distribution of BMI and expresses BMI as a SD score (SDS). This measure gives age- and gender-specific estimates of the distribution median, the coefficient of variation, and the degree of skew by a maximum likelihood fitting technique [12]. Systolic and diastolic BP were measured twice at the right arm after a 10 min rest in the supine position by using an automated oscillatory system (Dinamap Vital Signs Monitor, Model 1846 SX; Criticon Incorporated, Tampa, FL, USA).

The study was approved by the Hospital Ethics Committee, and informed consent was obtained from subjects' parents prior to assessment.

2.2. Laboratory Investigations. Blood samples were taken from each subject, after an overnight fast, for estimation of glucose, insulin, total cholesterol and high-density lipoprotein cholesterol (HDL-C), triglycerides, alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), TSH, FT4, FT3, and anti-TG and anti-TPO antibodies. All analyses were conducted by COBAS 6000. TSH, FT3, FT4, anti-TG and anti-TPO antibodies, and insulin were measured on cobas e 601 module (Electrochemiluminescence Technology, Roche Diagnostics), while the remaining analytes on cobas c 501 clinical chemistry module (Photometric Technology), according to the instructions of the manufacturer.

TSH was considered normal if it was between 0.4 and 4.0 mIU/L [13–16]. The reference intervals for FT3 and FT4 were 2.70–5.20 pg/mL and 0.80–1.90 ng/dL, respectively. Threshold values for anti-TG and anti-TPO antibodies were 115 and 34 IU/mL, respectively.

2.3. Definitions. Ultrasound examinations of the liver and of the thyroid were performed in real time using an Aplio XV (Toshiba Medical Systems) with 3.5–5-MHz convex transducers and tissue harmonics. Hepatic steatosis was defined by an appearance of hyperechoic liver parenchyma with tightly packed fine echoes and posterior beam attenuation. At ultrasound, thyroid size and morphology were evaluated with a high-resolution 7.5 MHz linear transducer, with the subjects sitting and their necks slightly extended. Thyroid volume was defined as enlarged according to the reference values for age, gender, and body surface area [17]. Thyroid tissue echogenicity was evaluated in a longitudinal scan of the thyroid lobes by a standardized comparison with the echogenicity of the adjacent muscles: sternohyoideus, sternothyroideus, and sternocleidomastoideus. Ultrasound scans were performed by a well-trained ultrasonographist who was unaware of the clinical and laboratory data.

For the American Heart Association (AHA) [18], MetS is diagnosed in the presence of any three of the following five constituent risks: central obesity as determined by WC, hypertension, low HDL-C values, elevated triglyceride values, and glucose impairment. We used the pediatric AHA definition [19], which is based on the AHA adult definition but uses pediatric reference standards for BP, WC, triglycerides, and HDL-C. Thus, in our study, central obesity was defined as a WC ≥90th percentile for age and gender; hypertriglyceridemia as triglycerides ≥90th percentile for age and gender; low HDL-C as concentrations ≤10th percentile for age and gender; elevated BP as systolic or diastolic BP ≥90th percentile for age, gender, and height percentile; and impaired fasting glucose as glucose ≥5.6 mmol/L. IR was determined by a homeostasis model assessment of insulin resistance (HOMA-IR) [20]. We considered HOMA-IR values ≥90th percentile for age and gender of those previously observed in a local population of healthy normal-weight children as an indicator of IR [21].

2.4. Statistical Analysis. Statistical analyses were performed using the SPSS package. Data are expressed either as frequencies or means with 95% confidence intervals (CIs). Distributions of continuous variables were examined for skewness and kurtosis and were logarithmically transformed, when appropriate. Geometric means are reported for TSH, FT3, FT4, total cholesterol and HDL-C, triglycerides, insulin, and HOMA-IR values. Differences between groups were tested for significance using independent sample t-test for quantitative variables, and chi-square test for qualitative

Table 1: Baseline characteristics of 402 overweight/obese children according to serum TSH.

	Serum TSH		P value
	≤4 mIU/L (n = 314)	>4 mIU/L (n = 88)	
TSH, mIU/L	2.42 (2.33–2.52)	4.74 (4.52–4.95)	<0.0001
Age, years	10.7 (10.3–11.1)	10.9 (10.0–11.2)	0.46
Male gender, n (%)	175 (55.7)	55 (62.5)	0.35
Prepubertal, n (%)	159 (50.6)	58 (65.9)	0.56
Weight, Kg	61 (59–63)	60 (55–65)	0.70
Height, cm	149 (147–152)	149 (144–153)	0.80
BMI, Kg/m^2	26.3 (25.7–26.8)	26.3 (25.2–27.3)	0.75
BMI-SDS	2.0 (1.94–2.2)	2.0 (1.9–2.2)	0.80
Waist circumference, cm	87 (84–90)	89 (87–90)	0.26
Systolic blood pressure, mmHg	109 (107–110)	110 (107–112)	0.88
Diastolic blood pressure, mmHg	69 (68–70)	70 (67–72)	0.82
Triglycerides, mg/dL	99 (90–108)	119 (103–135)	<0.01
Total cholesterol, mg/dL	160 (156–165)	176 (166–186)	<0.01
HDL-C, mg/dL	47 (46–49)	45 (43–48)	0.30
Fasting glucose, mmol/L	4.71 (4.64–4.78)	4.67 (4.58–4.75)	0.48
Insulin, mU/L	16.0 (15.0–18.0)	19.3 (16.5–22.0)	<0.05
HOMA-IR values	3.62 (3.22–4.0)	4.0 (3.4–4.6)	<0.05
ALT, IU/L	31 (28–35)	52 (36–68)	<0.01
GGT, IU/L	17 (16–19)	22 (18–26)	<0.01
FT3, pg/mL	4.26 (4.18–4.35)	4.35 (4.26–4.42)	0.14
FT4, ng/dL	1.18 (1.15–1.22)	1.18 (1.12–1.22)	0.76

Data are expressed as n (%), mean or geometric mean (95% confidence intervals).
TSH: thyroid-stimulating hormone; BMI: body mass index; BMI-SDS: BMI-SD score; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; FT3: free triiodothyronine; FT4: free thyroxine.

variables. Pearson's correlation and linear regression coefficients were used to examine the relationship between variables. Logistic regression analysis was performed to determine the independence of the association of TSH levels with hepatic steatosis, hepatic steatosis with elevated ALT, and metabolic variables, after adjustments for age, gender, pubertal status, and BMI-SDS (or WC) as well as FT3 and FT4 concentrations. Relationships between TSH levels and hepatic steatosis were also examined after adjustment for anthropometric and metabolic variables including hypertriglyceridemia, hypercholesterolemia, and insulin resistance. In the fully adjusted regression model, FT3 and FT4 levels were also included. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical and Laboratory Features of Study Population. The study group consisted of a consecutive series of 402 children fulfilling the selection criteria. Ninety-seven and 305 were overweight and obese, respectively. In Table 1 clinical, metabolic, and hormonal characteristics of overweight/obese children according to TSH level are summarized. Eighty-eight subjects (21.9%) had TSH above the normal range (>4.0 mIU/L). FT3 and FT4 were within the reference intervals in all subjects.

Compared to individuals with normal TSH, subjects with hyperthyrotropinemia showed significantly higher ALT, GGT, total cholesterol, triglycerides, insulin, and HOMA-IR values. Children with TSH ≤ 4.0 mIU/L and those with TSH over the normal range did not significantly differ in values of free thyroid hormones as well as in age, gender, pubertal status, or degree of total and central adiposity.

3.2. Thyroid Function Tests in relation to NAFLD and Metabolic Variables. Serum TSH concentrations were significantly higher in the 144 children with fatty liver compared to the 258 children with no fatty liver (3.20 (95% CI, 2.95–3.36) versus 2.65 (95% CI, 2.51–2.80); $P < 0.01$). The children with hepatic steatosis did not differ significantly from those with no hepatic steatosis in respect of FT3 (4.35 (95% CI, 4.26–4.48) versus 4.30 (95% CI, 4.14–4.40)) and FT4 (1.17 (95% CI, 1.13–1.21) versus 1.21 (95% CI, 1.17–1.24)).

As shown in Table 2, elevated TSH concentrations were significantly associated with an increased prevalence of hepatic steatosis, and hepatic steatosis with elevated ALT concentrations (serum ALT >25 IU/L for boys and >22 IU/L for girls [22]). Among the metabolic variables, the prevalence of hypertriglyceridemia, IR, elevated total cholesterol, and MetS (considered as a single clinical entity) was significantly higher in the 88 subjects with elevated TSH concentrations than in the 314 subjects with normal TSH concentrations.

Table 2: Prevalence of hepatic steatosis, hepatic steatosis with elevated ALT, metabolic syndrome, and its components according to serum TSH among 402 overweight/obese children.

	Serum TSH		P value
	≤4 mIU/L (n = 314)	>4 mIU/L (n = 88)	
Hepatic steatosis	101 (32.2)	43 (48.9)	<0.01
Hepatic steatosis with elevated ALT	73 (23.2)	31 (35.2)	<0.01
Central obesity	148 (47.1)	41 (46.6)	0.58
Elevated blood pressure	23 (7.3)	6 (6.8)	0.78
Hypertriglyceridemia	69 (22.0)	35 (39.8)	<0.01
Low HDL-C	57 (18.2)	17 (19.3)	0.20
Elevated total cholesterol	94 (29.9)	39 (44.3)	<0.01
Glucose ≥5.6 mmol/L	8 (2.5)	2 (2.3)	0.35
Insulin resistance	89 (28.3)	39 (44.3)	<0.01
Metabolic syndrome	32 (10.2)	17 (19.3)	<0.05

Data are presented as n (%).
TSH: thyroid-stimulating hormone; HDL-C: high-density lipoprotein cholesterol; ALT: alanine aminotransferase.

Table 3: Adjusted odds ratios (and 95% CIs) of hepatic steatosis, hepatic steatosis with elevated ALT, metabolic syndrome, and its components among 402 overweight/obese children.

	Serum TSH		P value
	≤4 mIU/L (n = 314)	>4 mIU/L (n = 88)	
Hepatic steatosis			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	2.10 (1.22–3.60)	<0.01
Hepatic steatosis with elevated ALT			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	2.42 (1.29–4.51)	<0.01
Central obesity			
Adjusted OR (95% CI)[+]	1.00 (referent)	1.32 (0.57–3.01)	0.52
Elevated blood pressure			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	1.31 (0.41–4.17)	0.58
Hypertriglyceridemia			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	2.58 (1.39–4.78)	<0.01
Low HDL-C			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	1.97 (0.98–3.98)	0.063
Elevated total cholesterol			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	2.23 (1.30–3.84)	<0.01
Glucose ≥5.6 mmol/L			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	0	
Insulin resistance			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	2.62 (1.45–4.74)	<0.01
Metabolic syndrome			
Adjusted OR (95% CI)[+,*]	1.00 (referent)	2.55 (1.13–5.76)	<0.05

[+] Age, gender, pubertal status, and BMI-SDS as well as FT3 and FT4 were included in the model; [*] similar results were obtained when adjusting for WC (instead of BMI-SDS).
TSH: thyroid-stimulating hormone; OR: odds ratio; CI: confidence interval; ALT: alanine aminotransferase; HDL-C: high-density lipoprotein cholesterol; BMI-SDS: body mass index-SD score; FT3: free triiodothyronine; FT4: free thyroxine; WC: waist circumference.

Table 3 shows the multivariate-adjusted associations between TSH and hepatic steatosis, as well as metabolic variables. High TSH was associated with an increased odds of having hepatic steatosis, hepatic steatosis with elevated ALT, hypertriglyceridemia, elevated total cholesterol, and IR as well as MetS, after adjustment for age, gender, pubertal status, and BMI-SDS (or WC) as well as FT3 and FT4. When a stepwise multivariate regression analysis included variables such as age, gender, pubertal status, BMI-SDS, hypertriglyceridemia, hypercholesterolemia, IR, and FT3 and FT4, the association between hepatic steatosis and TSH levels remained statistically significant (OR, 1.96 (95% CI, 1.10–3.75); $P < 0.05$). In this model, other covariates independently associated with hepatic steatosis were hypertriglyceridemia (2.73 (95% CI, 1.51–4.92); $P < 0.01$) and IR (2.37 (95% CI, 1.38–4.06); $P < 0.01$).

No association was found between FT4 and hepatic steatosis as well as MetS and its parameters after controlling for age, gender, pubertal status, and BMI-SDS (or WC).

4. Discussion

To the best of our knowledge, our study is the first to assess the relationships between thyroid function and hepatic steatosis in a large consecutive series of young participants. First, we found that in overweight/obese children and adolescents hyperthyrotropinemia was significantly associated with hepatic steatosis, after adjustments for age, gender, pubertal status, and degree of obesity. Second, we also found a significant association between elevated TSH levels and metabolic variables (including hypertriglyceridemia and IR which are usually altered in fatty liver). Finally, multivariate regression analysis showed that mild abnormalities in thyroid function were associated with hepatic steatosis independently of metabolic risk factors.

Previous studies have investigated the association between NAFLD (or surrogate markers of NAFLD) and thyroid function but solely in adult or elderly patients [4–10, 23]. The study by Targher et al. involving a large cohort of unselected adult outpatients was the first to report a strong association between thyroid function tests and serum liver enzyme activity concentrations [23]. In particular, Targher et al. found a significant positive relationship between serum TSH, ALT, and GGT activities throughout the normal and high TSH ranges, and a similar inverse relationship between FT4 and serum liver enzyme activity concentrations [23]. Notably, these results did not change after adjustment for a broad spectrum of potential confounders, such as gender, age, fasting glucose and lipid parameters. Unfortunately, that study did not provide information on medication use, lifestyle characteristics (i.e., daily alcohol consumption), obesity status, and degree of insulin resistance among study participants. The recent data from a large-scale population-based study [9], the Study of Health in Germany (SHIP), partly agree with those from Targher et al. [23]. In the SHIP study, there was a significant inverse association between FT4 concentration and hepatic steatosis (defined by the presence of a hyperechogenic ultrasound pattern of the liver and increased ALT concentrations), whereas TSH and FT3 concentrations were not consistently associated with hepatic steatosis, thus suggesting that overt but not subclinical hypothyroidism (SH) might be associated with hepatic steatosis [9]. In a cross-sectional study performed among 878 euthyroid elderly Chinese who took their annual healthy examination, the prevalence rate of ultrasound-diagnosed NAFLD showed an increasing trend as serum TSH level increased, while the rate showed a decreasing trend as serum FT4 level increased [6]. Finally, in a recent cross-sectional study involving 4,648 health check-up subjects [7], Chung et al. found that the prevalence of ultrasound-diagnosed NAFLD and abnormal ALT levels increased steadily with increasing grades of hypothyroidism (for NAFLD, subclinical: 29.9% and overt: 36.3%; for abnormal ALT, 20.1% and 25.9%, $P < 0.001$, resp.). SH was defined by Chung et al. as a serum TSH level over 4.1 mIU/L with normal FT4 concentration (0.7–1.8 ng/dL), while overt hypothyroidism as a FT4 level less than 0.7 ng/dL. An 1 IU/L increase of natural logged TSH was associated with a 20% increase in the prevalence of NAFLD, independently of known risk factors such as age, gender, BMI, WC, triglyceride, HDL-C, hypertension, and diabetes [7].

The published literature investigating the association between thyroid function and NAFLD has also included patients with biopsy-proven NAFLD. Liangpunsakul and Chalasani retrospectively found that the prevalence of hypothyroidism was significantly higher in adult patients with NASH when compared with age-, gender-, race-, and body weight-matched adults without liver disease attending the general medical clinics [4]. In that study, however, data on thyroid function tests were not available and cases were defined solely based on use of synthetic T4 replacement therapy, therefore suggesting a high frequency of participants with overt hypothyroidism. Likewise, in a case control study involving 256 adult patients with NAFLD and 430 age-, gender-, race-, and BMI-matched adult outpatients with normal liver tests and no evidence of acute or chronic liver disease, Pagadala et al. retrospectively found that subjects with hypothyroidism were 2.1 (95% CI, 1.1–3.9, $P = 0.02$) and 3.8 (95% CI, 2–6.9, $P < 0.001$) times more likely to have NAFLD and NASH, respectively [8]. After adjusting for diabetes, hypertension, BMI, and hyperlipidemia, subjects with hypothyroidism were still found to be 2.1 times (95% CI, 1.1–3.9, $P = 0.022$) more likely to have NAFLD than those without hypothyroidism. In addition, hypothyroidism was significantly higher in patients with NASH compared to those with no NASH [8]. However, that study again did not include laboratory data regarding thyroid function, and subjects were defined as having hypothyroidism if they carried a clinical diagnosis of hypothyroidism and were on thyroid replacement therapy. Yet, in a study involving 69 euthyroid adult patients with primary NAFLD [10], Carulli et al. retrospectively found that when biopsy-proven NASH patients were compared to those with no NASH, high TSH levels, independently of MetS and IR, were predictors of NASH, but the possibility of selection bias was raised in that study as well as in the previous ones [4, 5, 8] because of liver biopsy.

Against that background, the strengths of our study are the careful clinical characterization of the participants with their risk factor profiles, and by pediatric standards a fairly large study sample. Compared to previous studies [4–10, 23], our data differ in several design features including demographic and clinical characteristics (young versus adult or older participants with more advanced forms of the disease), inclusion and exclusion criteria, data collection (prospective versus retrospective), study selection (consecutive versus nonconsecutive series), population details (sufficient versus insufficient), study design (cohort versus case control), or participant recruitment (hospital-based versus population-based study). Nonetheless, our results as a whole allow us to confirm and expand on the findings of previous reports. In a population of overweight/obese children and adolescents, even slightly elevated serum levels of TSH are associated with an increased risk for NAFLD.

There are several underlying mechanisms which may substantiate the relationship between thyroid dysfunction and hepatic steatosis. Thyroid hormones influence all major metabolic pathways, and thyroid dysfunction, especially hypothyroidism, has been associated with IR [24, 25], dyslipidemia [26, 27], and obesity [28, 29], all of which play an important role in the development of NAFLD. IR in the setting of hypothyroidism has been documented [25] and is associated with decreased responsiveness of glucose uptake in muscle and adipose tissue to insulin, as well as decreased glycogen synthesis in skeletal muscle in both animal and human studies [24, 25, 30, 31]. These effects were alleviated by thyroid replacement [24]. Recently, IR has been shown to also exist in SH and to be comparable to that of clinical hypothyroidism [32]. Another explanation for the association between thyroid function and NAFLD is that hypothyroidism is associated with abnormal lipid values [27]. Hypothyroidism primarily causes elevation in cholesterol and low-density lipoproteins but it also affects the synthesis, mobilization and degradation of all aspects of lipid metabolism [26]. The elevation of triglycerides in hypothyroid subjects is caused by a reduced removal rate of triglycerides from plasma due to a decrease in the activity of hepatic triglyceride lipase [26, 33]. The effects of SH on serum lipids values are less clear. While some studies have demonstrated that serum triglycerides, lipid subparticle size, and low-density lipoprotein cholesterol (LDL-C) oxidizability may be altered in patients with SH, others have not shown any effect on these lipid alterations [34]. In agreement with previous studies in children [35, 36], we found that elevated TSH was significantly associated with hypertriglyceridemia as well as IR, independent of total and central obesity. Finally, hypothyroidism has been reported to modulate mitochondrial nitric oxide synthesis and alter mitochondrial inner membrane composition and permeability which alters respiratory gene expression and mitochondrial oxygen uptake [37]. Such abnormalities would result in increased ADP concentration and generation of reactive oxygen species [38].

This study has a few potential limitations. First, we had no histopathological data. Nonetheless, liver biopsies in a large study collective such as our cohort are difficult to perform, also for ethical reasons, as no specific therapy follows histologic diagnosis of NAFLD apart from recommending lifestyle modification which is generally advised to all obese children. Conversely, ultrasonography is by far the most common way of diagnosing hepatic steatosis in clinical practice. Second, it is cross-sectional; thus, our data are associations and do not prove causality. As opposed to healthy controls, serum TSH concentrations have been consistently found to be higher in obese subjects [13, 39]. Unlike TSH, data regarding the circulating levels of free thyroid hormones are discrepant between different studies, which reported either increased or decreased serum concentrations of FT3 [13, 28, 40, 41], with normal or decreased FT4/FT3 ratios [28, 41]. Because in some studies TSH fell with weight loss [42], it has been suggested that higher TSH in obese subjects is simply a metabolic adaption to excess body fat. However, weight and/or fat loss does not predictably decrease TSH and T3 [43–45]. Others have suggested that obesity-related SH, characterized by an increased serum TSH concentration with normal concentrations of the thyroid hormones, may be associated with dyslipidemia, IR, subclinical inflammation, and increased risk for coronary heart disease [46, 47]. In the study by Aeberli et al. who prospectively examined the associations between changes in thyroid function, IR, and other metabolic risk factors in obese children undergoing weight and fat loss in a well-controlled 8-week inpatient program [35], changes in TSH did not correlate with losses of weight, fat, or lean tissue (as assessed by dual energy X-ray absorption), but they significantly correlated with fasting insulin and HOMA-IR, independently of body weight and body composition. Yet, in that study [35], at baseline variation of TSH, but not variations in body weight or body fat, was a significant predictor of triglycerides, total cholesterol, and LDL-C. Baseline TSH was also an independent predictor of fasting insulin and HOMA-IR. In accordance with Aeberli et al. [35], we found that elevated TSH is a significant predictor of lipid and glucose dysmetabolism as well as of hepatic steatosis, independently of the degree of total and central obesity. Nonetheless, the possibility that thyroid hormones and NAFLD share common genetic or environmental influences accounting for the observed association cannot be discounted [48]. It is also possible that hepatic steatosis affects thyroid function rather than the other way around [48]. A longitudinal study with a cohort of children with and without hepatic steatosis at baseline would help clarify this.

Conflict of Interests

No potential conflict of interests relevant to this paper was reported.

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