Dietary walnut oil modulates liver steatosis in the obese Zucker rat

Anja Fink · Corinna E. Rüfer · Julie Le Grandois · Alexander Roth · Dalal Aoude-Werner · Eric Marchioni · Achim Bub · Stephan W. Barth

Received: 26 June 2013 / Accepted: 31 July 2013 / Published online: 13 August 2013 © The Author(s) 2013. This article is published with open access at Springerlink.com

Abstract

Purpose Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. We aimed to clarify the impact of dietary walnut oil versus animal fat on hepatic steatosis, representing the initial step of multistage pathogenesis of NAFLD, in Zucker obese rats.

Methods Zucker lean ad libitum (a.l.), Zucker obese a.l. or Zucker obese pair fed (p.f.) to the lean received isocaloric diets containing 8 % walnut oil (W8), W14 or 14 % lard (L14) (n = 10/group). Body weight, clinical serology, liver weight, lipid content and fatty acid composition and hepatic lipid metabolism-related transcripts were evaluated.

Results Compared to lean, Zucker obese a.l. and p.f. showed hepatic triacylglyceride (TAG) accumulation. In Zucker obese p.f., W14 compared to W8 and L14 reduced liver lipids, TAG as well as hepatic omega-6 (n-6)/n-3 ratio and SCD activity index [(C18:0 + C18:1)/C18:0 ratio] paralleled by decreased lipoprotein lipase mRNA in obese p.f. and elevated microsomal triglyceride transfer protein mRNA in lean and obese. Further, W14 elevated the fasting blood TAG and reduced cholesterol levels in obese.

Conclusions In our model, consumption of W14 inhibited hepatic lipid accumulation along with modulated hepatic gene expression implicated in hepatic fatty acid influx or lipoprotein assembly. These results provide first indication that dietary lipids from walnut oil are modulators of hepatic steatosis as the initial step of progressive NAFLD pathogenesis.

Keywords NAFLD · Lard · SFA · MUFA · PUFA

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common public health problem worldwide with an increased prevalence during the last three decades [1–3]. The prevalence of this disorder is strongly associated with several risk factors of the metabolic syndrome (MetS) such as obesity, dyslipidemia, hypertension and type 2 diabetes [4–7]. Therefore, NAFLD is regarded as the hepatic manifestation of the MetS [1, 8]. NAFLD, a multistage and progressive disease, ranges from lipid accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with a necroinflammatory component (non-alcoholic steatohepatitis; NASH) and might finally progresses to liver cirrhosis [5, 6, 9].

Liver triacylglyceride (TAG) levels are the result of the balance between influx of fatty acids, de novo lipogenesis, TAG synthesis and delivery by very low density lipoprotein (VLDL) assembly [10–13]. Thus, the accumulation of TAG in the steatotic liver reflects a difference between the rate at which fatty acids reach hepatocytes, or are
synthesized therein, and the rate at which they are metabolized, stored or assembled [10, 12, 13]. Further, this initial expansion of ectopic lipid levels during steatosis development tends to be associated with progressive metabolic dysregulation and, in particular, altered hepatic insulin resistance and the development of inflammation.

Until now, there has no specific therapy been developed to directly treat the pathogenesis and progression of NAFLD. Instead, indirect approaches are pursued by treating major risk factors of NAFLD. However, this treatment strategy is complex, because not a single but various risk factors influence and associate the induction and progression of NAFLD. Among others, mainly the developing insulin resistance and lipid dysmetabolism promote the pathological hepatic TAG accumulation [5, 6]. Hence, the cornerstones of the therapy as well as prevention of NAFLD are to ameliorate the metabolic risk factors by reducing body weight and improving insulin sensitivity and hyperlipidemia [6, 9].

The enhanced prevalence of NAFLD is also driven by changes of dietary habits and primarily increased consumption of dietary fat. Recent studies have further indicated that not solely the quantity of dietary fat, but also fatty acid composition is a risk-determining factor for NAFLD: Patients with NAFLD not only showed a higher intake of saturated fatty acids (SFA), but also increased omega-6 (n-6) polyunsaturated fatty acids (PUFA) consumption while n-3 PUFA uptake is reduced, leading to a significant shift toward increased dietary n-6/n-3 ratio [3, 8, 14].

Recent literature provides indications that these dietary changes might also lead to endogenous alterations in the hepatic fatty acid composition toward an increase in the n-6/n-3 PUFA ratio [15, 16], which associates with the development of an adverse metabolic profile and contribute to the pathogenesis of NAFLD [16]. Further, animal studies have demonstrated that an excess of n-6 PUFA in the liver is associated with a proinflammatory [3, 17] as well as steatogenic state with pronounced hepatic fat accumulation [18–20]. Given these observations, a dietary approach changing the n-6/n-3 PUFA ratio in the diet might be effective enough to change hepatic n-6/n-3 PUFA ratio in a way that positively influence NAFLD-related hepatic pathogenesis [21–23].

Nuts and seeds share a fatty acid profile which is characterized by low amounts of SFA and high content of monounsaturated fatty acids (MUFA) and PUFA [24]. Among the numerous nut varieties, recent research has focused on walnuts (Juglans regia). One reason for this might be the “optimal” 4:1 ratio of n-6/n-3 PUFA and additionally the high content of tocopherols (mainly γ-tocopherol), phytosterols, polyphenolic antioxidants (ellagitannins) and fiber [25, 26]. With regard to a significant bioactivity, recent investigations have shown that a walnut-rich diet improved hyperlipidemia [26–30], type 2 diabetes [31, 32], cardiovascular disease [33–35], ameliorated the antioxidative status [36, 37] and provided general parenchyma-protecting effects in the liver [30, 38]. However, to our best knowledge, no data are published regarding bioactivity of walnut on NAFLD mainly focusing on the initial step of liver steatosis.

Thus, the aim of the present study was to investigate the effect of different amounts of walnut oil versus lard on hepatic lipid metabolism in a rodent steatosis model and identify hepatic pathways which might be implicated in any observed bioactivity. Therefore, we have targeted hepatic lipid content, serum lipids and sICAM, as an artherogenic and proinflammatory marker. Additionally, we focused on the expression of genes involved in hepatic lipid metabolism to explore underlying mechanisms.

Materials and methods

Phospholipid analysis of walnut oil

Standard solutions of soy lecithin-mix standard (certified: 14.41 % phosphatidylcholine (PC), 12.06 % phosphatidylethanolamine (PE) and 9.64 % phosphatidylinositol (PI)) from Spectral Service GmbH (Cologne, Germany) were prepared at six concentrations between 0.1 and 1.1 mg/mL. Sand (Fontainebleau) was provided by VWR (Strasbourg, France).

The walnut oil (Öhlmühle Walz, Oberkirch, Germany) has been produced by a traditional two-step process performed in a hydraulic press driven by water power. The first step consists on a cold press of walnut seeds at 100 bar, followed by a 400 bar step at 40 °C. In subsequent to the pressing procedure, the oil is filtered through a rough filter mesh and bottled. For phospholipid (PL) analysis, 75 mg of oil samples were dissolved in 1 mL of CHCl3, transferred onto a preparative silica gel column (35 cm × 2 cm i.d., 15 g of Si 60 silica gel, particle size 40–63 μm, Geduran, Merck) which was pre-activated with 10 mL of CHCl3. Elution was performed at a flow rate of 4 mL/min. First, neutral lipids such as triacylglycerols and carotenoids were removed by 250 mL of CHCl3. Then, PL were eluted with 200 mL of CH3OH/1M aqueous formic acid (adjusted to pH = 3 with triethylamine) (98:2, v/v) mixture. The solvent was removed by rotary evaporation, and the residue containing pure PL was stored at −20 °C for the chromatographic separation.

A chromatographic system, made of a 616 controller, a 2424 ELS detector and a 717 Plus autosampler (Waters, Saint-Quentin-Fallavier, France) controlled with Empower 2 software (Waters, Saint-Quentin-Fallavier, France) was used to analyze PL classes. High-purity nitrogen from a nitrogen generator (Domnik Hunter, Villefranche-sur-Saône, France) was used as a nebulizing gas.
gas at a pressure of 310 kPa. The drift tube temperature was set at 45 °C. PL were separated into their respective classes (PE, PI, PC) using a 150 × 3 mm, 3 μm Luna normal phase column (Phenomenex, Le Pecq, France). The flow rate of mobile phase was 0.5 mL/min, and separations were performed at room temperature using a 20-min linear gradient ranging from CHCl3/CH3OH (88/12, v/v) to CHCl3/CH3OH/1M aqueous formic acid (adjusted to pH = 3 with triethylamine) (28/60/12, v/v/v). Each extract was dissolved in a mixture chloroform/methanol (2/1, v/v), filtered through a 0.45-μm filter (Macherey–Nagel, Hoerdt, France) to eliminate particles and injected (20 μL) in the chromatographic system. PL classes were identified by comparing their retention times with those obtained under the same analytical conditions with standards. Quantification of each PL class was performed based on a quadratic model of external calibration obtained by using standard solutions.

Phytosterol analysis of walnut oil

Solvents used for extraction and purification were of analytical grade. Ethanol (95 %), diethyl ether, n-hexane and cyclohexane were purchased from LPCR (Strasbourg, France). Analytical-grade potassium hydroxide (LPCR, Strasbourg France) was used for saponification. 1-methyl imidazole (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and N-methyl-N-trimethylsilyl-heptafluorobutyramide (MSHFBA) (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were used for sterols silylation. Sterols analysis was adapted from EN ISO 12228 [39].

One mL of internal standard (betulin solution at 1 mg/mL) is added to 250 mg of walnut oil in a round-bottom flask. This sample is dissolved in 5 mL of potassium hydroxide, 0.5 M in ethanol (95 %) and heated under reflux for 15 min. After cooling, the whole saponified sample is subjected to column chromatography on an alumina column (10 g) preconditioned with 20 mL of ethanol 95 %. Elution of unsaponifiable matter was first performed with 5 mL ethanol, then with 30 mL diethyl ether. Eluents were combined and evaporated under vacuum. Residue was dissolved in 1 mL cyclohexane/diethyl ether (9:1, v/v) before being subjected to solid-phase extraction on Chromabond SiOH cartridge (3 mL, 500 mg, Macherey–Nagel, Hoerdt, France) preconditioned with 5 mL cyclohexane. Washing step was performed with 5 mL of cyclohexane/diethyl ether (9:1, v/v). Finally, sterols elution was achieved using 8 mL of cyclohexane/diethyl ether (1:1, v/v). The eluent was evaporated under vacuum. Sterols were silylated using 50 μL of 1-methyl imidazole and 1 mL MSHFBA, before injection (1 μL) in GC-FID. Separation was made by a CP Sil 8-CB column (50 m, 0.25 mm, 0.25 μm, Agilent Technologies). Helium of high purity (99.9995 %) was used as carrier gas at a flow rate of 1.4 mL/min. The injector was set to 250 °C and the flame ionization detector to 320 °C. The column was set at 80 °C, held for 1 min, and raised to 280 °C (rate of 10 °C/min). The temperature was then raised to 308 °C (rate of 0.5 °C/min) and then to 320 °C (rate of 2 °C/min). The final temperature was maintained for 3 min. Peaks were identified by a comparison with standards and to relative retention times (RRT). Quantification was performed using betuline as internal standard, considering response factors between sterols and betuline as equal to 1.

Tocopherol analysis of walnut oil

The method used for analysis was adapted from Skrivanova et al. [40]. About two grams of walnut oil were diluted in 25 mL of HPLC grade n-hexane (LPCR, Strasbourg, France). The solution is filtered through a 0.45 μm PTFE membrane (Macherey–Nagel, Hoerdt, France) before injection. Separation of tocopherols (α-, β-, γ- and δ-tocopherol) is performed on a Lichrospher Si 60 column (250 × 4.6 mm, 5 μm, Merck, Darmstadt, Germany) with an isocratic mobile phase based on n-hexane/2-propanol (98:2, v/v). Detection was done by fluorimetry, with excitation and emission wavelengths set to 284 and 330 nm, respectively. Quantification of each tocopherol was performed using a calibration curve prepared with tocopherol standards (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Animals and diets

Obese (fa/fa; n = 60) and lean (Fa/+; n = 30) female Zucker rats were purchased from Charles River Laboratories (Lyon, France) at the age of 6 weeks. All animals were housed in a temperature- and humidity-controlled animal facility under ambient temperature of 21 ± 2 °C, 55–65 % of relative humidity and a 12–12 h light–dark cycle. During the first week of adaption, all rats were provided ad libitum (a.l.) tap water and a standard experimental diet AIN93G with 7 % corn oil (Ssniff, Soest, Germany). In subsequent to the adaptation, rats were randomly allocated to one of the following groups: lean a.l., obese pair fed (p.f.) or obese a.l. The group of the obese p.f. rats were fed the same amount of food consumed at the previous day by the age-matched lean counterparts. This pair feeding concept has been conducted, because the total energy intake is a major determinant for obesity (Table 5), dyslipidemia (Fig. 2a) as well as hepatic steatosis (Fig. 1a, b). As the pair feeding group receives the same amount of isocaloric diets and thus the same amount of energy compared to the lean group, this confounding factor has been controlled by this concept. Thus, any observed differences between lean and obese p.f. are determined by genotype, while
Fig. 1 Effects of different diets with 8 % walnut oil (black), 14 % walnut oil (white) or 14 % lard (gray) on a relative liver weight, b hepatic TAG and c hepatic cholesterol content in lean ad libitum (a.l.), obese pair fed (p.f.) or obese a.l. Zucker rats. Data are expressed as mean ± SD (n = 9–10). The letters a, b and c describe the significant differences (p < 0.05) of the phenotypes with the same feeding. Significant differences within phenotypes are labeled by asterisk (p < 0.05)

### Table 1 Composition of experimental diets (%; w/w)

| Experimental diets | 8 % Walnut oil | 14 % Walnut oil | 14 % Lard |
|--------------------|----------------|-----------------|---------|
| Casein             | 20.00          | 20.00           | 20.00   |
| Corn starch mod    | 38.74          | 23.74           | 23.74   |
| Maltodextrin       | 13.20          | 13.20           | 13.20   |
| Saccharose         | 10.00          | 10.00           | 10.00   |
| Lignocellulose     | 5.00           | 0.30            | 0.30    |
| l-cystein          | 0.30           | 0.30            | 0.30    |
| Vitamin-AIN        | 1.00           | 1.00            | 1.00    |
| Mineral nutrients  | 2.50           | 3.50            | 3.50    |
| Butylhydroxytoluol | 0.01           | 0.01            | 0.01    |
| Choline            | 0.25           | 0.25            | 0.25    |
| Fat                | 8.00           | 14.00           | 14.00   |
| ME, Atwater (MJ/kg)| 16.60          | 16.60           | 16.60   |

### Table 2 Fatty acid composition of experimental diets (% of total fatty acids)

| Fatty acid methyl esters (% of total fatty acids) | 8 % Walnut oil | 14 % Walnut oil | 14 % Lard |
|--------------------------------------------------|----------------|-----------------|---------|
| C12:0 (lauric acid)                              | 0.0            | 0.0             | 0.2     |
| C14:0 (myristic acid)                            | 0.2            | 0.1             | 2.6     |
| C16:0 (palmitic acid)                            | 9.8            | 9.4             | 34.7    |
| C17:0 (margaric acid)                            | 0.1            | 0.1             | 0.7     |
| C18:0 (stearic acid)                             | 5.8            | 5.8             | 20.5    |
| C20:0 (arachidic acid)                           | 0.1            | 0.1             | 0.1     |
| C22:0 (behenic acid)                             | 0.0            | 0.0             | 0.0     |
| SFA                                              | 16.0           | 15.5            | 58.7    |
| C14:1 (myristoleic acid)                         | 0.0            | 0.0             | 0.2     |
| C16:1 (palmitoleic acid)                         | 0.2            | 0.2             | 2.8     |
| C18:1n-9t (elaidic acid)                         | 0.1            | 0.1             | 0.2     |
| C18:1n-9c (oleic acid)                           | 27.5           | 27.3            | 26.9    |
| C20:1 (gondoic acid)                             | 0.3            | 0.3             | 1.2     |
| C24:1n-9 (nervonic acid)                         | 0.0            | 0.0             | 0.1     |
| MUFA                                             | 28.1           | 27.9            | 31.3    |
| C18:2n-6t (linolaic acid)                         | 0.3            | 0.3             | 0.0     |
| C18:2n-6c (linoleic acid)                        | 39.2           | 39.6            | 8.8     |
| C18:3n-6 (γ-linolenic acid)                       | 0.5            | 0.5             | 0.3     |
| C18:3n-3 (α-linolenic acid)                       | 15.9           | 16.1            | 0.5     |
| C20:2n-6 (eicosadienoic acid)                     | 0.0            | 0.0             | 0.2     |
| C20:3n-6 (dihomo γ-linolenic acid)                | 0.0            | 0.0             | 0.1     |
| C20:3n-3 (eicosatrienoic acid)                    | 0.0            | 0.0             | 0.0     |
| n-6/n-3 PUFA ratio                               | 2.5:1          | 2.5:1           | 18:1    |

 Springer
differences between obese p.f. and obese a.l. are due to the different intake of amount of food. All groups were further randomly subdivided into one of the three intervention groups (n = 10) receiving either an isocaloric diet based on AIN93G containing 8 % (w/w) walnut oil (W8; Öhlmühle Walz, Oberkirch), 14 % walnut oil (W14) or 14 % (w/w) lard (L14) (ssniff, Soest, Germany) for 10 weeks (Table 1). The fatty acid composition of diets and the analytical data of walnut oil phytosterols, tocopherols and phospholipids are summarized in Tables 2 and 3, respectively. Food intake was measured daily and body weight four times a week. At the end of the feeding period, the animals were fasted overnight, deeply anesthetized by carbon dioxide and killed by decapitation. Liver tissues were collected and stored at –80 °C. All animal experiments were approved by the Animal Care Committee of the Regional Administrative Authority, Karlsruhe (35-9185.81/G-89/09), and all animal care and handling were conducted in accordance with the guidelines of the German law on animal care.

Blood parameters

Blood was collected into serum monovettes (Sarstedt, Nümbrecht, Germany), and serum was prepared from clotted blood by centrifugation (2,500 × g, 10 min) and stored at –30 °C until further analysis. Serum cholesterol (CHOD-PAP, Roche) and TAG (GPO-PAP, Roche) were measured using enzymatic assay kits. Serum level of soluble intercellular adhesion molecule (sICAM)-1 (R&D systems, Wiesbaden, Germany) was measured using a rat ELISA kit.

Liver lipid and fatty acid analyses

Lipid extraction of 2 g liver tissues was carried out according to Hara and Radin [41] with slight modifications. The liver tissue was homogenized in 18 mL of hexane/isopropanol (3/2; v/v) (Carl Roth, Karlsruhe, Germany) containing 0.01 % butylhydroxytoluol (BHT; Merck, Darmstadt, Germany). After sonification and addition of 12 mL aqueous sodium sulfate (Merck), samples were placed on a horizontal shaker for overnight extraction. The samples were centrifuged and hexane overlayer was removed to a fresh pre-weighted vial. The remainder bottom layer was again extracted with 18 mL of hexane/isopropanol (7/2; v/v) containing 0.01 % BHT, and after centrifugation, the hexane overlayer was given to the vial already containing the hexane layer of the initial extraction. After evaporation under a stream of nitrogen gas, total lipid content within sample vials was weighted for gravimetric estimation of total liver fat. Fatty acids were analyzed by gas chromatography (GC). Fatty acid methyl esters (FAME) were prepared by transesterification of total lipids with TMSH. 50 mg of the lipid extract dissolved in 1 mL dichloromethane and aliquots of 10 μL were used for FAME analysis. After addition of 100 μL of 90 mg/L methyl-nonadecanoate as internal standard, 30 μL of TMSH, as well as 70 μL methanol containing 1 % BHT, the organic extract was evaporated to complete dryness under a stream of nitrogen. The residue was dissolved in 100 μL TMSH and 500 μL methanol, stirred in the dark overnight and subjected to GC analysis. GC analysis was carried out on a gas chromatograph with mass spectrometric detection (GC-MS-QP-2010 Ultra, Shimadzu, Kyoto, Japan) using split/splitless injection. Chromatographic separation of FAME were achieved on a fused silica capillary column with a non-bonded cyanopropyl-type phase (SP-2560, 75 m × 0.18 mm ID, 0.14 μM film thickness, Supelco, Taufkirchen, Germany) using a helium carrier gas flow of 0.9 mL/min and a linear temperature gradient (100 °C for 5 min, then 4 °C/min to 230 °C and hold at 230 °C for 25 min). The injector port temperature was set to 230 °C. The temperatures of transfer line and ion source were set to 250 and 200 °C, respectively. The injection volume was 1 μL with a split of 1:5. Individual methyl esters were identified and quantified using a standard mixture of 37 FAME (37 Component FAME Mix, Supelco, Taufkirchen, Germany). As based on the fatty acid quantification, hepatic stearoyl CoA desaturase (SCD-1) activity index was calculated by the (C18:0 + C18:1)/C18:0 ratio. Hepatic cholesterol and TAG were measured as previously described for serum samples.

### Table 3 Analysis of phytosterols, tocopherols and phospholipids in walnut oil (mg/100 g)

| Phytosterol                  | (mg/100 g) |
|------------------------------|------------|
| Brassicasterol               | 0.00       |
| Campesterol                  | 8.89       |
| Cholesterol                  | 0.39       |
| d5-Avenasterol               | 1.09       |
| d7-Avenasterol               | 0.00       |
| d7-Campesterol               | 0.00       |
| d7-Stigmasterol              | 49.46      |
| Sitostanol                   | 17.91      |
| Sitosterol                   | 163.48     |
| Stigmasterol                 | n.d.       |
| Phytosterols total           | 241.21     |
| α-Tocopherol                 | 2.394      |
| γ-Tocopherol                 | 45.450     |
| δ-Tocopherol                 | 5.044      |
| Tocopherols total            | 52.890     |
| Phosphatidylethanolamine     | 22.5       |
| Phosphatidylinositol         | n.d.       |
| Phosphatidylcholine          | n.d.       |
| Phospholipids total          | 22.5       |

n.d. not detectable

Eur J Nutr (2014) 53:645–660
Real-time quantitative PCR

Total RNA was isolated from the liver tissue using a commercial kit according to the manufacturer’s instruction (Total RNA and protein isolation kit, Macherey–Nagel, Düren, Germany). cDNA was prepared by reverse transcription of 2 µg total RNA using the Transcriptor First Strand cDNA Synthesis Kit and oligo(dT) primers (Roche). Samples were stored at −20 °C until further use. Semi-quantitative real-time PCR was carried out using the Light Cycler480 Instrument (Roche). Primer and probe sequences were designed by Universal Probe Library (Roche) as listed in Table 4. The reaction mixture contained 5 µL cDNA, corresponding to 50 ng total RNA, 0.5 µM of each primer, 1 µM probe and Light Cycler480 Probe Master Mix (2× conc.) (Roche). The PCR conditions were as follows: 10 min of initial denaturation at 95 °C followed by 45 amplification cycles each at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s with a terminal cooling period of 10 s at 40 °C. The analysis was carried out with the Light Cycler480 Software (Roche) using the relative quantification ΔΔCT-method and normalized by beta-actin as reference gene.

Statistical analysis

Due to high heterogeneity of variances between lean and obese, a statistical model, namely, generalized least squares ANOVA (GLS-ANOVA) was chosen that is capable of handling unequal variances for the different groups. The independent variables for the two-factorial ANOVA were “phenotype” and “food”, resulting in a 3 × 3 factorial model. As a first step, we calculated a simple model which assumes equal variances within factors “phenotype” and “food”. Subsequently, this simple model was then modified by adjusting the variance structure for “phenotype” and “food”. A likelihood ratio test was used to determine if the variance-adapted models fitted better than the simple model. When main effects or interactions were found significant, a post hoc test (Tukey–Kramer) for pairwise comparisons was applied. The p values that were given in the text and figures result from these post hoc tests. To test for the assumptions that have to be met for the GLS-ANOVA, we made plots to visually inspect residuals. Studentized residuals were plotted against fitted values to assess homoscedasticity. QQ-plots were used to test normal distribution of the residuals. Further, both plots were used to identify outliers. All calculations were carried out by R 2.15.2 [42]. GLS-ANOVAs were calculated by R package nlme [43].

Results

Analysis of dietary lipids and experimental diets

Fatty acid spectra of walnut oil and lard-containing diets were characteristic for the respective lipid. While the PUFA linoleic and α-linolenic are the major fatty acids in walnut oil, lard mainly contained the SFA palmitic and stearic acids. The resulting ratio of n-6/n-3 PUFA were 2.5:1 and 18:1 in walnut oil and lard-containing experimental diets, respectively (Table 2). The analytical results of walnut oil phospholipid content (Table 3) showed significant concentrations only for phosphatidylethanolamine (22.5 mg/100 g), while no trace of phosphatidylinositol was recovered, and phosphatidylcholine, the major phospholipid in common foods, was present only in trace amounts and therefore was not quantified here. Further, sitosterol (163 mg/100 g) and d7-stigmasterol (49.5 mg/100 g) represent the major phytosterol constituents in walnut oil, while content of γ-tocopherol (45.5 mg/100 g) was highest compared to α-(2.4 mg/100 g) and δ-tocopherols (5.0 mg/100 g) (Table 3).
Table 5  Body weight, weight gain and food intake during the experiment in lean ad libitum (a.l.), obese pair fed (p.f.), and obese a.l. Zucker rats receiving diets with 8 % walnut oil (W8), 14 % walnut oil (W14) and 14 % lard (L14)

| Experimental diets | Lean a.l. | Obese p.f. | Obese a.l. |
|--------------------|-----------|------------|------------|
| Initial body weight (g) | W8 136 ± 7a | 189 ± 10b | 196 ± 13b |
|                     | W14 139 ± 7a | 196 ± 8b  | 203 ± 10b |
|                     | L14 140 ± 9a | 191 ± 8b  | 198 ± 11b |
| Final body weight (g)  | W8 246 ± 19a | 349 ± 21b | 395 ± 21c |
|                      | W14 245 ± 20a | 356 ± 34b | 406 ± 36c |
|                      | L14 242 ± 15a | 337 ± 27b | 386 ± 38c |
| Weight gain (g/week)  | W8 11.2 ± 1.3a | 16.7 ± 1.7b | 20.3 ± 1.7c |
|                      | W14 11.0 ± 1.6a | 16.8 ± 3.4b | 20.3 ± 3.5c |
|                      | L14 10.6 ± 1.4a | 15.3 ± 2.8b | 18.8 ± 3.6c |
| Food intake (g/day)   | W8 13.2 ± 0.4a | 13.2 ± 0.1a | 16.4 ± 1.2b |
|                      | W14 12.8 ± 0.6a | 13.2 ± 0.1a | 16.3 ± 0.9b |
|                      | L14 13.3 ± 0.3a | 13.4 ± 0.1a | 16.5 ± 0.5b |

Data are expressed as mean ± SD (n = 9–10). Values in a common row of the identical intervention labeled by different letters are significantly different between phenotypes.

Significant differences within phenotypes are labeled by * (p < 0.05 compared to W8)

Body weight and food intake

As shown in Table 5, body weight, weight gain and food intake were significantly affected by genotype as well as the feeding regimen, namely the pair feeding versus ad libitum in obese rats. Obese p.f. and obese a.l. rats showed a significantly (p < 0.001) higher initial and final body weight than lean rats with obese a.l. rats weighing more than obese p.f. rats (p < 0.001). Further, the mean body weight gain was significantly lower in lean than in obese a.l. (p < 0.001) and obese p.f. (p < 0.001) Zucker rats and obese p.f. rats gained less weight throughout the experiment when compared to obese a.l. (p < 0.001). The mean food intake during the entire experiment was approximately 25 % higher in obese a.l. than in lean (p < 0.001) and the matched obese p.f. rats (p < 0.001). As a result of the established pair feeding regimen, statistical evaluation between the lean and obese p.f. showed equal food intake between obese p.f. and lean rats.

Besides the observed dependency of general parameters on genotype and dietary energy intake, the amount and quality of different dietary fats did not significantly influence the food intake, final body weight and mean weight gain (Table 5).

Liver lipids

Irrespective of pair or ad libitum feeding, the Zucker obese rats developed liver steatosis as reflected by higher liver weights as well as liver total lipid and TAG content as compared to the lean. In detail, the relative liver weight (Fig. 1a) and TAG contents (Fig. 1b) in obese p.f. and a.l. rats were significantly higher than in lean. Pair feeding resulted in a significantly lower relative liver weight (p < 0.05) when compared to ad libitum fed obese rats (Fig. 1a). With the exception of the W8 group, the hepatic TAG contents in pair fed were lower than in ad libitum fed rats (Fig. 1b). Moreover, the liver tissue of obese p.f. rats contained significantly higher (p < 0.01) cholesterol than the liver of lean rats, and obese a.l. rats showed higher cholesterol contents than obese p.f. rats, except of the W8 group (Fig. 1c).

In lean rats, none of the different dietary fats led to significant group differences regarding the relative liver weight or any of the analyzed lipid parameters in the liver. However, in obese p.f. rats, consumption of both 14 % fat diets significantly reduced (p < 0.05) the relative liver weight when compared to W8. Further, the liver weight in W14 obese a.l. rats was significantly reduced (p < 0.001) compared to L14 rats (Fig. 1a).

In accordance to the remainder liver parameters, also, the total liver lipid content was unaffected by any diet in lean Zucker rats. However, feeding W14 compared to W8 significantly reduced (p < 0.01) the total liver lipid content in obese p.f. rats. This drop in liver lipids seems to be specific to walnut, as it was not observed in L14 (data not shown). Further, the observed reduction in lipid content by W14 is a consequence mainly of the reduced hepatic TAG as the liver TAG content in obese p.f. rats was significantly reduced by W14 (p < 0.001) but not by L14 as compared to W8 group. In lean and obese a.l. rats, a W14 diet led to a significantly (p < 0.05) lower hepatic TAG compared to the L14 group (Fig. 1b). The hepatic cholesterol content was also affected by the different lipid diets. In obese p.f.
rats, W14 and L14 diets reduced the hepatic cholesterol content significantly \((p < 0.01)\) compared to the W8 group, and obese a.l. W14 rats had also a lower cholesterol \((p < 0.05)\) content compared to the W8 group (Fig. 1c).

Liver fatty acids

The fatty acid patterns of the experimental diets were distinct and characteristic to walnut oil and lard as detailed in Table 2. Walnut oil has a low content of SFA and a high content of PUFA, especially n-3 PUFA, whereas a high content of SFA and a low content of PUFA are characteristic for lard (Table 2).

Irrespective of the different experimental diets, obese p.f. and obese a.l. Zucker rats generally showed significantly lower \((p < 0.001)\) hepatic SFA- and PUFA-contents compared to the lean Zucker rats, while the MUFA content was significantly higher \((p < 0.001)\) in obese p.f. and a.l. rats (Table 2). In accordance to the higher MUFA content in obese livers, also, the SCD activity index \([\text{C18:0 + C18:1}/\text{C18:0 ratio}]\) was significantly higher in obese animals (Fig. 4).

Further, the different diets differentially affected the SFA, MUFA and PUFA content in the liver of the respective feeding groups. As depicted in Table 6, the hepatic SFA levels were unaffected by any diet in lean, obese p.f. and obese a.l. Although not statistically significant, the hepatic MUFA content tended to be decreased by W14 while MUFA content was highest under L14 feeding in all phenotypes. Accordingly, also the SCD activity index significantly decreased by W14 in obese p.f. and obese a.l., while L14 compared to W14 significantly increased the SCD activity index in all phenotypes (Fig. 4). While MUFA was highest in L14 groups, feeding L14 significantly decreased PUFA content compared to respective W8 and W14 groups. Further, in obese p.f. and obese a.l., but not in lean Zucker rats, the W14 diet significantly increased \((p < 0.001)\) the hepatic PUFA content compared to the respective W8 and the L14 groups. In detail, the W14 diet elevated the n-3 PUFA \(\alpha\)-linolenic acid \((\text{C18:3n-3})\), eicosapentaenoic acid \((\text{C20:5n-3})\) and docosahexaenoic acid \((\text{C20:6n-3})\) in the liver of obese p.f. and obese a.l. rats compared to rats with a W8 and L14 diet (not shown). In consequence, the ratio of n-6/n-3 decreased, although n-6 increased too. This drop in n-6/n-3 ratio by W14 was also observable in the lean but not as pronounced as in obese (Table 6).

Blood parameters

As expected, obese p.f. and obese a.l. Zucker rats developed a significant dyslipidemia reflected by a pronounced hypertriglyceridemia and hypercholesterolemia as compared to the lean (Fig. 2). Further, serum sICAM concentrations were significantly enhanced in obese p.f. and a.l. compared to lean Zucker rats, and obese a.l. showed significantly increased sICAM levels compared to obese p.f. (Fig. 3).

When compared to the W14-mediated lipid- and TAG-lowering effect in the liver of obese animals, walnut oil showed the opposite effect in plasma: In obese rats, W14 led to a significant \((p < 0.001)\) increase in fasting plasma

### Table 6: Fatty acid profile in liver samples from lean ad libitum (a.l.), obese pair fed (p.f.) and obese a.l. Zucker rats receiving diets with 8% walnut oil (W8), 14% walnut oil (W14) and 14% lard (L14)

| Fatty acid methyl ester (% of total fatty acids) | Experimental diets | Lean a.l. | Obese p.f. | Obese a.l. |
|-----------------------------------------------|--------------------|----------|-----------|-----------|
| SFA                                           | W8                 | 52.7 ± 5.6<sup>a</sup> | 41.9 ± 1.7<sup>b</sup> | 40.7 ± 0.9<sup>c</sup> |
|                                               | W14                | 52.7 ± 5.2<sup>a</sup> | 41.4 ± 1.3<sup>b</sup> | 39.0 ± 0.9<sup>c</sup> |
|                                               | L14                | 52.9 ± 3.1<sup>a</sup> | 43.0 ± 3.5<sup>b</sup> | 41.1 ± 1.7<sup>c</sup> |
| MUFA                                          | W8                 | 11.1 ± 5.7<sup>a</sup> | 48.3 ± 0.6<sup>b</sup> | 48.8 ± 1.5<sup>b</sup> |
|                                               | W14                | 7.7 ± 1.6<sup>a</sup>  | 40.1 ± 3.4<sup>b</sup> | 39.6 ± 5.2<sup>b</sup> |
|                                               | L14                | 22.1 ± 6.5<sup>a</sup> | 51.5 ± 4.8<sup>b</sup> | 54.2 ± 3.1<sup>b</sup> |
| PUFA                                          | W8                 | 36.2 ± 3.2<sup>a</sup> | 9.8 ± 2.0<sup>b</sup>  | 10.6 ± 1.1<sup>b</sup> |
|                                               | W14                | 38.6 ± 3.6<sup>a</sup> | 18.6 ± 2.9<sup>b</sup> | 21.3 ± 4.8<sup>b</sup> |
|                                               | L14                | 25.0 ± 6.7<sup>a</sup> | 5.5 ± 3.4<sup>b</sup>  | 4.7 ± 1.8<sup>b</sup>  |
| n-6 PUFA                                      | W8                 | 29.9 ± 1.7<sup>a</sup> | 9.2 ± 1.8<sup>b</sup>  | 9.9 ± 0.8<sup>b</sup>  |
| n-3 PUFA                                      | W8                 | 21.9 ± 5.3<sup>a</sup> | 5.3 ± 3.3<sup>b</sup>  | 4.6 ± 1.7<sup>b</sup>  |
|                                               | W14                | 6.0 ± 1.4<sup>a</sup>  | 0.6 ± 0.2<sup>b</sup>  | 0.6 ± 0.2<sup>b</sup>  |
|                                               | L14                | 3.0 ± 1.5<sup>a</sup>  | 0.2 ± 0.1<sup>b</sup>  | 0.2 ± 0.1<sup>b</sup>  |
| n-6/n-3 PUFA ratio                            | W8                 | 5:1                  | 15:1                  | 17:1                  |
|                                               | W14                | 5:1                  | 9:1                   | 9:1                   |
|                                               | L14                | 7:1                  | 27:1                  | 23:1                  |
This TAG-enhancing effect seems to be not specifically deserved to the intake of W14, but also L14 showed a significant \((p < 0.05)\) rise in plasma TAG when compared to W8 (Fig. 2a) in obese a.l. Zucker rats. The cholesterol-lowering effect is also apparent in lean and seems to be specific to walnut oil as solely W14, but not L14 decreased plasma cholesterol compared to W8 (Fig. 2b). Besides the modification of plasma lipid metabolites, also, circulating sICAM levels have been affected by the different experimental diets. In both obese groups, W14 and L14 led to a significant reduction in serum sICAM concentrations compared to respective W8, while W14-mediated sICAM was significantly lower when compared to L14 (Fig. 3).

Spearman’s rank correlation indicated that the total liver lipid content is positively and significantly correlated with serum sICAM \((\rho = 0.97; p < 0.001)\), while liver and serum TAG are negatively correlated \((\rho = -0.49; p < 0.001)\).

Hepatic gene expression

Transcripts coding for key enzymes and transcription factors of the lipid metabolic pathway respecting factors which influence the fatty acid uptake, lipogenic and lipolytic regulators as well as transcripts coding for factors implicated in VLDL assembly were analyzed.
The gene expression of hepatic enzymes involved in hydrolysis of TAG to free fatty acids, namely lipoprotein lipase (LPL) showed significant phenotype differences between obese and lean Zucker rats. The liver LPL gene expression was up-regulated in obese p.f. and a.l. Zucker rats, except for obese p.f. W14, compared to respective lean groups (Fig. 5). The expression of peroxisome proliferator-activated receptor (PPAR)-\(\alpha\), a transcription factor involved in fatty acid oxidation was significantly higher in obese a.l. rats than in obese p.f. rats (Table 7). Amount of transcript coding for lipogenic enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) showed significantly enhanced mRNA levels in obese p.f. Zucker rats compared to lean and obese a.l. Zucker rats.

Table 7  Expression of key genes involved in hepatic lipid metabolism was determined by real-time PCR in liver samples lean ad libitum (a.l.), obese pair fed (p.f.) and obese a.l. Zucker rats receiving diets with 8 % walnut oil, 14 % walnut oil, or 14 % lard. Data are expressed as mean ± SD (n = 8–10). Values in a common row of the identical intervention containing labeled by different letters are significantly different between phenotypes. Significant differences within phenotypes are labeled by * (p < 0.05 compared to W8) or # (p < 0.05 compared to W14).

| Experimental diet | Lean a.l. | Obese p.f. | Obese a.l. |
|-------------------|----------|------------|------------|
| ACC/β-Actin       |          |            |            |
| W8                | 2.9 ± 2.8\(^a\) | 3.3 ± 1.7\(^b\) | 2.0 ± 0.6\(^a\) |
| W14               | 2.7 ± 2.6\(^a\) | 5.4 ± 1.8\(^b\) | 2.8 ± 1.1\(^a\) |
| L14               | 3.4 ± 3.2\(^a\) | 5.2 ± 2.4\(^b\) | 2.5 ± 1.1\(^a\) |
| ChREBP/β-Actin    |          |            |            |
| W8                | 1.3 ± 0.5\(^a\) | 0.7 ± 0.2\(^b\) | 0.6 ± 0.1\(^c\) |
| W14               | 1.2 ± 0.3\(^a\) | 0.8 ± 0.2\(^b\) | 0.7 ± 0.1\(^c\) |
| L14               | 1.1 ± 0.4\(^a\) | 0.7 ± 0.2\(^b\) | 0.6 ± 0.2\(^c\) |
| DGAT/β-Actin      |          |            |            |
| W8                | 1.2 ± 0.3\(^a\) | 0.8 ± 0.3\(^ab\) | 0.9 ± 0.4\(^b\) |
| W14               | 1.3 ± 0.4\(^a\) | 1.4 ± 0.5\(^ab\) | 1.0 ± 0.4\(^b\) |
| L14               | 1.4 ± 0.4\(^a\) | 1.3 ± 0.6\(^ab\) | 0.9 ± 0.5\(^b\) |
| FAS/β-Actin       |          |            |            |
| W8                | 1.7 ± 2.1\(^a\) | 2.7 ± 1.8\(^b\) | 1.6 ± 0.7\(^a\) |
| W14               | 1.7 ± 1.7\(^a\) | 4.9 ± 1.8\(^b\) | 2.1 ± 0.8\(^a\) |
| L14               | 2.0 ± 1.8\(^a\) | 4.3 ± 1.8\(^b\) | 1.9 ± 0.7\(^a\) |
| GPAm/β-Actin      |          |            |            |
| W8                | 0.9 ± 0.2\(^a\) | 2.2 ± 1.9\(^b\) | 1.3 ± 0.5\(^c\) |
| W14               | 1.2 ± 0.8\(^a\) | 5.0 ± 2.3\(^b\) | 1.7 ± 1.2\(^c\) |
| L14               | 1.2 ± 0.6\(^a\) | 3.2 ± 2.8\(^b\) | 1.2 ± 0.7\(^c\) |
| ICAM/β-Actin      |          |            |            |
| W8                | 0.6 ± 0.1\(^a\) | 1.6 ± 0.4\(^b\) | 1.6 ± 0.4\(^b\) |
| W14               | 0.8 ± 0.3\(^a\) | 1.2 ± 0.2\(^a\) | 1.9 ± 0.7\(^b\) |
| L14               | 0.6 ± 0.2\(^a\) | 1.3 ± 0.3\(^b\) | 1.6 ± 0.3\(^b\) |
| PPAR-α/β-Actin    |          |            |            |
| W8                | 2.0 ± 0.6\(^a\) | 1.8 ± 0.6\(^a\) | 2.3 ± 0.6\(^b\) |
| W14               | 2.0 ± 0.7\(^a\) | 1.8 ± 0.8\(^a\) | 2.6 ± 1.0\(^b\) |
| L14               | 2.1 ± 0.7\(^a\) | 1.5 ± 0.4\(^a\) | 2.1 ± 0.5\(^b\) |
| PPAR-γ/β-Actin    |          |            |            |
| W8                | 1.2 ± 0.6\(^a\) | 6.4 ± 4.1\(^b\) | 5.2 ± 1.6\(^b\) |
| W14               | 1.1 ± 0.4\(^a\) | 5.3 ± 2.1\(^b\) | 6.5 ± 4.3\(^b\) |
| L14               | 1.1 ± 0.5\(^a\) | 5.8 ± 2.2\(^b\) | 5.9 ± 2.6\(^b\) |
| SREBP1c/β-Actin   |          |            |            |
| W8                | 0.8 ± 0.4\(^a\) | 1.7 ± 1.1\(^b\) | 1.9 ± 0.6\(^b\) |
| W14               | 1.1 ± 0.4\(^a\) | 2.3 ± 0.8\(^b\) | 2.0 ± 0.7\(^b\) |
| L14               | 1.1 ± 0.7\(^a\) | 2.0 ± 0.8\(^b\) | 2.0 ± 0.8\(^b\) |
(Table 7). Furthermore, lipogenic transcription factors PPAR-γ and sterol regulatory element-binding protein (SREBP)-1c were up-regulated, whereas carbohydrate responsive element-binding protein (ChREBP) was down-regulated in obese Zucker rats compared to the lean (Table 7). Amount of mitochondrial glyceral-3-phosphate acyltransferase (GPAm) mRNA, which codes for a key enzyme implicated in TAG esterification, was significantly higher in obese p.f. rats compared to obese a.l and lean rats. Diglyceride acyltransferase (DGAT), a key enzyme for the esterification of TAG, was significantly induced in lean rats compared to obese a.l. rats. Furthermore, the expression of microsomal TAG transfer protein (MTTP), a key factor regulating hepatic TAG export by VLDL synthesis and assembly, was significantly down-regulated in obese p.f. W14 group, the hepatic ICAM gene expression was significantly up-regulated in all obese compared to lean Zucker rats (Table 7).

The intervention with the different experimental diets differentially modulated the expression of LPL in liver tissue. In obese p.f., both diets containing 14 % fat content led to a significant reduction of LPL mRNA in the liver compared to obese p.f. W8 Zucker rats. In contrast, no effects were observed in lean Zucker rats (Fig. 5). Spearman’s rank correlation showed that the total liver lipid content is positively and significantly correlated with the hepatic LPL mRNA content (ρ 0.785; p < 0.001).

Irrespective of the phenotype, the MTTP gene expression has been significantly increased in all W14 groups as compared to groups receiving W8 or L14 diets (Fig. 5). All other transcript levels of ACC, FAS, SREBP-1c, ChREBP, PPAR-γ, PPAR-α, GPAm, DGAT, MTTP, ICAM were unaffected by any diet (Table 7).

Discussion

The key finding of this study stated that the quality and the quantity of dietary lipids differentially influence hepatic and circulating lipid metabolites in vivo. In detail, a high intake of walnut oil decreased hepatic TAG while fasting serum TAG levels were increased in obese Zucker rats. The decreased hepatic TAG concomitantly appeared with significant changes in fatty acid patterns in the liver and a reduced SCD activity index as well as reduced/normalized n-6/n-3 ratio in the liver. These qualitative and quantitative changes in lipid contents were associated with decreased hepatic LPL mRNA in obese p.f. rats and increased MTTP mRNA irrespective of the phenotype. Finally, a diet high in walnut oil significantly reduced the atherogenic and inflammatory marker sICAM in obese Zucker rats.

The development of efficient (diet-based) prevention and therapeutic options for NAFLD is based on a clear understanding of the etiology and mechanisms of this condition, which in turn is limited by the quality of the study model. NAFLD in lab rodents can be induced and promoted by a wide variety of factors, initially leading to changes in hepatic lipid deposition. These include diets which are high in fat or carbohydrates (e.g., fructose) or methionine and choline deficient (MCD) diets. The diet-induced NAFLD models are completed by various genetic models; the most commonly used are represented by the leptin and/or leptin receptor variants. All these currently available animal models of NAFLD are associated with various drawbacks in that they do not or only partially reflect the real picture of human NAFLD in terms of etiology, pathogenesis and disease mechanisms. For example, most of the diet-induced NAFLD models also implicate potential confounding factors such as high energy intake (by high-fat and/or high-carbohydrate diets) or the high-fructose, lipid or deficient-(MCD) intake of particular macronutrients. On the other side, genetic models carrying a single mutation do not share the same etiology as multifactorially/multigenetically generated human NAFLD, although clinical parameters related to NAFLD show identical aberrations [44–48]. Based on this knowledge about respective model-associated advantages and disadvantages, we chose the obese Zucker rat, integrated this model into an energy-controlled pair feeding design and further used isocaloric diets irrespective of the different fat contents. According to this experimental design, the pair feeding groups received the amount of diet adjusted to the respective lean a.l. group which received approximately 75 % of dietary calories as compared to the obese a.l. fed groups. This concept combined with isocaloric diets irrespective of fat content enabled us to analyze a “pure” intervention effect of dietary lipid quality and/or quantity on NAFLD-associated metabolic aberrations largely excluding the mentioned potential confounders such as variable energy intake.

The present study shows that an isocaloric diet with a high content of walnut oil (14 % w/w) not solely mediated anti-steatotic effects in obese rats, but it also influenced the hepatic fatty acid composition.

It has been described that various dietary fats with different fatty acid compositions differentially affect the hepatic TAG accumulation [49, 50]. In vivo studies have already shown that dietary fats from fish oil rich in long-chain n-3 PUFA resulted in a reduction in hepatic TAG concentration [49–52], which has recently been shown to be associated with a preferred incorporation of long-chain n-3 PUFA into hepatic phospholipids [53]. Further, Chechi et al. [2] have demonstrated that a flax oil diet, rich in α-linolenic acid, also reduced the hepatic TAG in obese Zucker rats. The intervention with the different experimental diets excluding the mentioned potential confounders such as high energy intake (by high-fat and/or high-carbohydrate diets) or the high-fructose, lipid or deficient-(MCD) intake of particular macronutrients. On the other side, genetic models carrying a single mutation do not share the same etiology as multifactorially/multigenetically generated human NAFLD, although clinical parameters related to NAFLD show identical aberrations [44–48]. Based on this knowledge about respective model-associated advantages and disadvantages, we chose the obese Zucker rat, integrated this model into an energy-controlled pair feeding design and further used isocaloric diets irrespective of the different fat contents. According to this experimental design, the pair feeding groups received the amount of diet adjusted to the respective lean a.l. group which received approximately 75 % of dietary calories as compared to the obese a.l. fed groups. This concept combined with isocaloric diets irrespective of fat content enabled us to analyze a “pure” intervention effect of dietary lipid quality and/or quantity on NAFLD-associated metabolic aberrations largely excluding the mentioned potential confounders such as variable energy intake.

The present study shows that an isocaloric diet with a high content of walnut oil (14 % w/w) not solely mediated anti-steatotic effects in obese rats, but it also influenced the hepatic fatty acid composition.

It has been described that various dietary fats with different fatty acid compositions differentially affect the hepatic TAG accumulation [49, 50]. In vivo studies have already shown that dietary fats from fish oil rich in long-chain n-3 PUFA resulted in a reduction in hepatic TAG concentration [49–52], which has recently been shown to be associated with a preferred incorporation of long-chain n-3 PUFA into hepatic phospholipids [53]. Further, Chechi et al. [2] have demonstrated that a flax oil diet, rich in α-linolenic acid, also reduced the hepatic TAG in obese Zucker rats. The intervention with the different experimental diets excluding the mentioned potential confounders such as high energy intake (by high-fat and/or high-carbohydrate diets) or the high-fructose, lipid or deficient-(MCD) intake of particular macronutrients. On the other side, genetic models carrying a single mutation do not share the same etiology as multifactorially/multigenetically generated human NAFLD, although clinical parameters related to NAFLD show identical aberrations [44–48]. Based on this knowledge about respective model-associated advantages and disadvantages, we chose the obese Zucker rat, integrated this model into an energy-controlled pair feeding design and further used isocaloric diets irrespective of the different fat contents. According to this experimental design, the pair feeding groups received the amount of diet adjusted to the respective lean a.l. group which received approximately 75 % of dietary calories as compared to the obese a.l. fed groups. This concept combined with isocaloric diets irrespective of fat content enabled us to analyze a “pure” intervention effect of dietary lipid quality and/or quantity on NAFLD-associated metabolic aberrations largely excluding the mentioned potential confounders such as variable energy intake.

The present study shows that an isocaloric diet with a high content of walnut oil (14 % w/w) not solely mediated anti-steatotic effects in obese rats, but it also influenced the hepatic fatty acid composition.

It has been described that various dietary fats with different fatty acid compositions differentially affect the hepatic TAG accumulation [49, 50]. In vivo studies have already shown that dietary fats from fish oil rich in long-chain n-3 PUFA resulted in a reduction in hepatic TAG concentration [49–52], which has recently been shown to be associated with a preferred incorporation of long-chain n-3 PUFA into hepatic phospholipids [53]. Further, Chechi et al. [2] have demonstrated that a flax oil diet, rich in α-linolenic acid, also reduced the hepatic TAG in obese Zucker rats.
However, the results presented here let us suggest that a TAG by suppressing hepatic VLDL assembly [60–63].

molecules and also catalyzing hepatocyte VLDL assembly. hepatic receptors (e.g., PPARs), enzymes and transport also capable of being signaling molecules directly affecting being substrate for hepatic TAG synthesis, fatty acids are effect. These results might be an indication that besides ad libitum fed rats, also, the high intake of lard shows this serum TAG in energy-controlled obese rats. In obese oil was paralleled by a significant elevation of fasting plasma TAG which might also be the result of the ApoE deficiency [22, 51]. Riediger et al. [22] reported decreased plasma TAG content in vivo [23, 57]. Based on these data, we suggest that the observed bioactivity of high walnut oil intake in obese rats might be caused by rather than randomly associated with the observed increased levels of hepatic PUFA, especially n-3 PUFA. The predominating n-3 PUFA of walnut oil, α-linolenic acid (Table 2), is endogenously converted into eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) by the Δ-6- and Δ-5-desaturase and elongase-5 and -2 enzyme systems, respectively. Although the efficacy of this conversion of α-linolenic acid into EPA and DHA is not quantitative, but has been estimated below 21 % [58, 59], significant two to fourfold enrichment of hepatic EPA or DHA by W14 compared to W8 was obvious (data not shown) and paralleled by a general improvement of n-6/n-3 ratio under anti-steatotic feeding with walnut oil.

Epidemiological studies have reported that besides its impact on hepatic lipid homoeostasis, a low n-6/n-3 PUFA ratio is also capable of improving the blood lipid profile [22, 51]. Riediger et al. [22] reported decreased plasma TAG levels by flax oil diet with a low n-6/n-3 PUFA ratio. A high-fat diet containing PUFA compared to high SFA diet led to a decreased concentration of TAG in the serum of C57BL/6J mice [51]. However, recently published data show that a high intake of walnut oil did not change plasma TAG which might also be the result of the ApoE deficiency in this model [54].

As opposed to these latter-published data in our study, the drop in hepatic TAG content by high intake of walnut oil was paralleled by a significant elevation of fasting serum TAG in energy-controlled obese rats. In obese ad libitum fed rats, also, the high intake of lard shows this effect. These results might be an indication that besides being substrate for hepatic TAG synthesis, fatty acids are also capable of being signaling molecules directly affecting hepatic receptors (e.g., PPARs), enzymes and transport molecules and also catalyzing hepatocyte VLDL assembly.

It has been established in several studies in human and animal experiments that dietary n-3 PUFA decrease plasma TAG by suppressing hepatic VLDL assembly [60–63]. However, the results presented here let us suggest that a high intake of walnut oil rather increased than decreased VLDL secretion as reflected by elevated plasma concentration of fasting TAG in steatotic animals.

Increasing fasting plasma lipids might be an indication for an elevating insulin resistance characterized by diabetic dyslipidemia [64]. However, in the present study, changes of insulin resistance causing the observed hypertriglyceridemia may be excluded, as plasma insulin and glucose as well as adiponectin were not significantly affected by walnut oil in our study mobilized and reduced the hepatic lipid content as discussed below.

While low levels of hepatic n-3 PUFA are associated with the development of liver steatosis in NAFLD patients [18, 57], a supplementation with n-3 PUFA decreased hepatic TAG content in vivo [23, 57]. Based on these data, we suggest that the observed bioactivity of high walnut oil intake in obese rats might be caused by rather than randomly associated with the observed increased levels of hepatic PUFA, especially n-3 PUFA. The predominating n-3 PUFA of walnut oil, α-linolenic acid (Table 2), is endogenously converted into eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) by the Δ-6- and Δ-5-desaturase and elongase-5 and -2 enzyme systems, respectively. Although the efficacy of this conversion of α-linolenic acid into EPA and DHA is not quantitative, but has been estimated below 21 % [58, 59], significant two to fourfold enrichment of hepatic EPA or DHA by W14 compared to W8 was obvious (data not shown) and paralleled by a general improvement of n-6/n-3 ratio under anti-steatotic feeding with walnut oil.

Epidemiological studies have reported that besides its impact on hepatic lipid homoeostasis, a low n-6/n-3 PUFA ratio is also capable of improving the blood lipid profile [22, 51]. Riediger et al. [22] reported decreased plasma TAG levels by flax oil diet with a low n-6/n-3 PUFA ratio. A high-fat diet containing PUFA compared to high SFA diet led to a decreased concentration of TAG in the serum of C57BL/6J mice [51]. However, recently published data show that a high intake of walnut oil did not change plasma TAG which might also be the result of the ApoE deficiency in this model [54].

As opposed to these latter-published data in our study, the drop in hepatic TAG content by high intake of walnut oil was paralleled by a significant elevation of fasting serum TAG in energy-controlled obese rats. In obese ad libitum fed rats, also, the high intake of lard shows this effect. These results might be an indication that besides being substrate for hepatic TAG synthesis, fatty acids are also capable of being signaling molecules directly affecting hepatic receptors (e.g., PPARs), enzymes and transport molecules and also catalyzing hepatocyte VLDL assembly.

It has been established in several studies in human and animal experiments that dietary n-3 PUFA decrease plasma TAG by suppressing hepatic VLDL assembly [60–63]. However, the results presented here let us suggest that a high intake of walnut oil rather increased than decreased VLDL secretion as reflected by elevated plasma concentration of fasting TAG in steatotic animals.

Increasing fasting plasma lipids might be an indication for an elevating insulin resistance characterized by diabetic dyslipidemia [64]. However, in the present study, changes of insulin resistance causing the observed hypertriglyceridemia may be excluded, as plasma insulin and glucose as well as adiponectin were not significantly affected by walnut oil in our study mobilized and reduced the hepatic lipid content as discussed below.

While low levels of hepatic n-3 PUFA are associated with the development of liver steatosis in NAFLD patients [18, 57], a supplementation with n-3 PUFA decreased hepatic TAG content in vivo [23, 57]. Based on these data, we suggest that the observed bioactivity of high walnut oil intake in obese rats might be caused by rather than randomly associated with the observed increased levels of hepatic PUFA, especially n-3 PUFA. The predominating n-3 PUFA of walnut oil, α-linolenic acid (Table 2), is endogenously converted into eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) by the Δ-6- and Δ-5-desaturase and elongase-5 and -2 enzyme systems, respectively. Although the efficacy of this conversion of α-linolenic acid into EPA and DHA is not quantitative, but has been estimated below 21 % [58, 59], significant two to fourfold enrichment of hepatic EPA or DHA by W14 compared to W8 was obvious (data not shown) and paralleled by a general improvement of n-6/n-3 ratio under anti-steatotic feeding with walnut oil.

Epidemiological studies have reported that besides its impact on hepatic lipid homoeostasis, a low n-6/n-3 PUFA ratio is also capable of improving the blood lipid profile [22, 51]. Riediger et al. [22] reported decreased plasma TAG levels by flax oil diet with a low n-6/n-3 PUFA ratio. A high-fat diet containing PUFA compared to high SFA diet led to a decreased concentration of TAG in the serum of C57BL/6J mice [51]. However, recently published data show that a high intake of walnut oil did not change plasma TAG which might also be the result of the ApoE deficiency in this model [54].

As opposed to these latter-published data in our study, the drop in hepatic TAG content by high intake of walnut oil was paralleled by a significant elevation of fasting serum TAG in energy-controlled obese rats. In obese ad libitum fed rats, also, the high intake of lard shows this effect. These results might be an indication that besides being substrate for hepatic TAG synthesis, fatty acids are also capable of being signaling molecules directly affecting hepatic receptors (e.g., PPARs), enzymes and transport molecules and also catalyzing hepatocyte VLDL assembly.

It has been established in several studies in human and animal experiments that dietary n-3 PUFA decrease plasma TAG by suppressing hepatic VLDL assembly [60–63]. However, the results presented here let us suggest that a high intake of walnut oil rather increased than decreased VLDL secretion as reflected by elevated plasma concentration of fasting TAG in steatotic animals.

Increasing fasting plasma lipids might be an indication for an elevating insulin resistance characterized by diabetic dyslipidemia [64]. However, in the present study, changes of insulin resistance causing the observed hypertriglyceridemia may be excluded, as plasma insulin and glucose as well as adiponectin were not significantly affected by walnut oil in our study mobilized and reduced the hepatic lipid content as discussed below.

While low levels of hepatic n-3 PUFA are associated with the development of liver steatosis in NAFLD patients [18, 57], a supplementation with n-3 PUFA decreased hepatic TAG content in vivo [23, 57]. Based on these data, we suggest that the observed bioactivity of high walnut oil intake in obese rats might be caused by rather than randomly associated with the observed increased levels of hepatic PUFA, especially n-3 PUFA. The predominating n-3 PUFA of walnut oil, α-linolenic acid (Table 2), is endogenously converted into eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) by the Δ-6- and Δ-5-desaturase and elongase-5 and -2 enzyme systems, respectively. Although the efficacy of this conversion of α-linolenic acid into EPA and DHA is not quantitative, but has been estimated below 21 % [58, 59], significant two to fourfold enrichment of hepatic EPA or DHA by W14 compared to W8 was obvious (data not shown) and paralleled by a general improvement of n-6/n-3 ratio under anti-steatotic feeding with walnut oil.

Epidemiological studies have reported that besides its impact on hepatic lipid homoeostasis, a low n-6/n-3 PUFA ratio is also capable of improving the blood lipid profile [22, 51]. Riediger et al. [22] reported decreased plasma TAG levels by flax oil diet with a low n-6/n-3 PUFA ratio. A high-fat diet containing PUFA compared to high SFA diet led to a decreased concentration of TAG in the serum of C57BL/6J mice [51]. However, recently published data show that a high intake of walnut oil did not change plasma TAG which might also be the result of the ApoE deficiency in this model [54].

As opposed to these latter-published data in our study, the drop in hepatic TAG content by high intake of walnut oil was paralleled by a significant elevation of fasting serum TAG in energy-controlled obese rats. In obese ad libitum fed rats, also, the high intake of lard shows this effect. These results might be an indication that besides being substrate for hepatic TAG synthesis, fatty acids are also capable of being signaling molecules directly affecting hepatic receptors (e.g., PPARs), enzymes and transport molecules and also catalyzing hepatocyte VLDL assembly.

It has been established in several studies in human and animal experiments that dietary n-3 PUFA decrease plasma TAG by suppressing hepatic VLDL assembly [60–63]. However, the results presented here let us suggest that a
Further, hepatic D random related to, but might be at least in part a W14-provoked decrease in hepatic TAG might not be suggest that under our experimental conditions of the not significantly reduced accordingly. Therefore, we index in steatotic obese, but SREBP1 mRNA levels were upregulated by SREBP-1 which was increased in our study rather than a high supply of dietary oleic acid [75, 76].

18:0 ratio in hepatocytes. Moreover, elevated hepatic TAG and therefore, changes of SCD activity modulate the 18:1/ enzyme of hepatic desaturation of stearic acid to oleic acid, the SCD enzyme activity. This enzyme is the rate-limiting part be responsible for the walnut oil-mediated decrease in the high intake of lard significantly reduced LPL gene expression.

Interestingly, our gene expression data further show that a high load with walnut oil, but not lard significantly increased MTTP gene expression in lean and obese Zucker rats. MTTP exerts a central role in VLDL assembly and secretion [10]. Biosynthesis of hepatic VLDL is critically dependent on the coordinated interactions of apolipoprotein B (apoB) and MTTP [72]. The knock-out of MTTP impairs the assembly and secretion of TAG-rich lipoproteins from the liver [73], while hepatic MTTP overexpression in lep-in-deficient mice resulted in an increased VLDL secretion and a decreased hepatic TAG content [74]. Summarizing these latter and our current findings, we suggest that the observed MTTP overexpression might in part be responsible for the walnut oil-mediated decrease in hepatic and increase in circulating TAG.

In addition to these results, we also found that the SCD-index is significantly higher in obese compared to respective lean groups. The SCD-index is an indirect indicator for the SCD enzyme activity. This enzyme is the rate-limiting enzyme of hepatic desaturation of stearic acid to oleic acid, and therefore, changes of SCD activity modulate the 18:1/18:0 ratio in hepatocytes. Moreover, elevated hepatic TAG levels and provoked lipogenesis were mainly mediated by upregulation of SCD activity and oleic acid biosynthesis rather than a high supply of dietary oleic acid [75, 76]. Further, hepatic Δ5- and Δ6-desaturases are known to be upregulated by SREBP-1 which was increased in our study in obese as compared to respective lean, too. In addition, we have shown that W14 significantly depressed the SCD-index in steatotic obese, but SREBP1 mRNA levels were not significantly reduced accordingly. Therefore, we suggest that under our experimental conditions of the W14-provoked decrease in hepatic TAG might not be randomly related to, but might be at least in part a consequence of reduced SCD-1 activity and endogenous oleic acid biosynthesis.

Collectively, the results of our study establish that the observed bioactivity of walnut oil can be attributed to multiple metabolic modulations, including an elevated TAG efflux by VLDL assembly. This, in combination with impaired lipolytic conversion of TAG-rich remnants and reduced hepatic oleic acid and subsequent reduced TAG synthesis results in subsequent reduced hepatic and elevated circulating TAG levels.

Besides the walnut oil-mediated modulation of hepatic and circulating TAG concentration, a high walnut-intake reduces total cholesterol as summarized in the framework of a meta-analysis [77], which has been confirmed more recently by a randomized cross-over study in free-living [78] and diabetic subjects [33]. The results derived from feeding studies confirmed the cholesterol-lowering effect of walnuts [79], while rodent data on the cholesterol-lowering effect are more inconsistent, showing either no effect [54] or an increased serum cholesterol concentration after feeding a 5 % (w/w) walnut oil diet in ApoE-deficient hypercholesterolemic mice [80]. Thus, our observations of a cholesterol-lowering effect of high walnut oil are in line with human data. As this effect has not been observed in high lard groups, the cholesterol-lowering effect might be either a result of the PUFA content of walnut oil [81], leading to a decreased cholesterol synthesis or a consequence of walnut oil phytosterols, especially sitosterol and campesterol (Table 3), which share structural similarity with cholesterol and thus reduce serum cholesterol by inhibition of cholesterol absorption [26, 82].

In addition to the reduced serum cholesterol, also, the hepatic cholesterol content is significantly decreased by high walnut oil and lard. Previous studies have shown that PUFA-rich diets suppress SREBP-1 which is a key transcriptional factor involved in up-regulation of cholesterol synthesis [83]. Accordingly, also in our study, the higher hepatic cholesterol content in obese well corresponded to elevated hepatic SREBP-1 mRNA levels in obese compared to the lean groups. However, the SREBP-1 gene expression did not significantly change, and also SREBP-1 target genes FAS and ACC remained constant, while high walnut oil significantly dropped serum cholesterol in all phenotypes. Thus, the observed reduction in serum cholesterol by walnut oil is regulated independently from hepatic SREBP-1 or changes in SREBP-1 expression due to post-transcriptional regulation, e.g., by changing the maturation of SREBP-1 protein, as described previously for PUFA-rich fish oil [83].

Patients with NAFLD have increased circulating level of atherosclerotic biomarkers like sICAM [84–86]. Although classically synthesized and secreted by vascular endothelial cells, Thakur et al. [84] suggested that circulating levels of
sICAM might serve as a useful marker of liver injury and steatohepatitis. They observed higher sICAM concentrations in subjects with NAFLD than in lean controls, which is in line with the observations in our animal model. Whether the circulating levels of sICAM also originate from the liver is most likely, as NASH livers express elevated amounts of ICAM mRNA [85]. In addition, Thakur et al. [84] described that the steatotic compared to the normal liver synthesize and probably also secrete elevated amounts of hepatic ICAM-1. Although not being reflected according to significant mRNA changes, our finding that a high intake of walnut oil to a significantly higher extend than lard significantly decreased serum sICAM in parallel with the observed loss of hepatic TAG further promotes the suggestion that circulating sICAM might originate from the liver and also relates to the grade of hepatic steatosis in our model.

In conclusion, our results provide evidence that walnut oil is capable of differentially modifying hepatic and systemic lipid homeostasis. Moreover, walnut oil also affects endocrine factors associated with atherosclerosis or chronic inflammation. Further, with regard to most parameters relevant to steatosis-associated dyslipidemia, the bioactivity of walnut oil is most probably distinct from that of similar high dose of lard and thus might be the result of the complex mixture of PUFA, but also phytosterols and/or tocopherols might contribute to this bioactivity as also γ-tocopherol has been shown to decrease liver TAG content in a rodent fatty liver model [87]. In this context, it has to be emphasized that whole foods might exert effects beyond those of identified single major ingredients [88] clearly opposing pharmaceutically driven concepts that favor the use of purified fatty acids for drug-based NAFLD intervention, as recently reviewed by Musso et al. [89]. Regarding this general view of favoring complex food instead of individual compounds, also the anti-prostate cancer bioactivity of whole walnut seeds could unexpectedly not be attributed to their fatty acids or tocopherol contents [79]. Perspectives, our work on walnut oil will be extended to the whole walnut seed, too, in order to elucidate the combinatory role of polyphenolic and lipophilic walnut ingredients with respect to their anti-steatotic bioactivity.

Acknowledgments We thank D. Haase, E. Hoch, C. Hoffmann, D. Kolbinger and A. Waldheiser for their excellent technical assistance and Dr. A. Jaudszus and Dr. R. Krüger for helpful discussions and careful revision of the manuscript. This work was performed in frame of the nutrhi-net-project, funded by the EC InterReg IVa Program.

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

1. Byrne CD, Olufadi R, Bruce KD, Cagampang FR, Ahmed MH (2009) Metabolic disturbances in non-alcoholic fatty liver disease. Clin Sci (Lond) 116:539–564
2. Chechi K, Yasui N, Ikeda K, Yamori Y, Cheema K (2010) Flax oil-mediated activation of PPAR-gamma correlates with reduction of hepatic lipid accumulation in obese spontaneously hypertensive/NDmcrc-p rats, a model of the metabolic syndrome. Br J Nutr 104:1313–1321
3. Molendi-Coste O, Legry V, Leclercq IA (2010) Dietary lipids and NAFLD: suggestions for improved nutrition. Acta Gastroenterol Belg 73:431–436
4. Day CP (2011) Non-alcoholic fatty liver disease: a massive problem. Clin Med 11:176–178
5. Della CC, Alisi A, Iorio R, Alterio A, Nobili V (2011) Expert opinion on current therapies for nonalcoholic fatty liver disease. Expert Opin Pharmacother 12:1901–1911
6. Lewis JR, Mohanty SR (2010) Nonalcoholic fatty liver disease: a review and update. Dig Dis Sci 55:560–578
7. Petta S, Muratore C, Craxi A (2009) Non-alcoholic fatty liver disease pathogenesis: the present and the future. Dig Liver Dis 41:615–625
8. Yki-Jarvinen H (2010) Nutritional modulation of nonalcoholic fatty liver disease and insulin resistance: human data. Curr Opin Clin Nutr Metab Care 13:709–714
9. Krawczyk M, Bonfante L, Portincasa P (2010) Nonalcoholic fatty liver disease. Best Pract Res Clin Gastroenterol 24:695–708
10. Musso G, Gambino R, Cassader M (2009) Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). Prog Lipid Res 48:1–26
11. Postic C, Girard J (2008) The role of the lipogenic pathway in the development of hepatic steatosis. Diabetes Metab 34:643–648
12. Vanni E, Bugianesi E, Kotronen A, De MS, Yki-Jarvinen H, Svegliati-Baroni G (2010) From the metabolic syndrome to NAFLD or vice versa? Dig Liver Dis 42:320–330
13. Cohen JC, Horton JD, Hobbs HH (2011) Human fatty liver disease: old questions and new insights. Science 332:1519–1523
14. Musso G, Gambino R, De MF, Cassader M, Rizzato M, Durazzo M, Faga E, Silli B, Pagano G (2003) Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology 37:909–916
15. Parker HM, Johnson NA, Burdon CA, Cohn JS, O’Connor HT, George J (2011) Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol. doi: 10.1016/j.jhep.2011.08.018
16. Toshimitsu K, Matsuura B, Ohkubo I, Niiya T, Furukawa S, Hiasa Y, Kawamura M, Ebihara K, Onji M (2007) Dietary habits and nutrient intake in non-alcoholic steatohepatitis. Hepatology 37:909–916
17. Wall R, Ross RP, Fitzgerald GF, Stanton C (2010) Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 68:280–289
18. Araya J, Rodrigo R, Videla LA, Thieleman L, Orellana M, Pettinelli P, Poniachik J (2004) Increase in long-chain polyunsaturated fatty acid n-6/n-3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. Clin Sci (Lond) 106:635–643
19. Xu J, Cho H, O’Malley S, Park JH, Clarke SD (2002) Dietary polyunsaturated fats regulate rat liver sterol regulatory element
binding proteins-1 and -2 in three distinct stages and by different mechanisms. J Nutr 132:3333–3339
20. Zivkovic AM, German JB, Sanyal AJ (2007) Comparative review of diets for the metabolic syndrome: implications for nonalcoholic fatty liver disease. Am J Clin Nutr 86:285–300
21. El-Badry ND, Othman R, Fitz E, Pierce GN, Suh M, Moghaddas MH (2008) Low n-6:n-3 fatty acid ratio, with fish- or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. Eur J Nutr 47:153–160
22. Sekiya M, Yahagi N, Matsuzaka T, Najima Y, Nakakuki M, Nagai R, Ishibashi S, Osuga J, Yamada N, Shimano H (2003) Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. Hepatology 38:1529–1539
23. Maguire LS, O’Sullivan SM, Galvin K, O’Connor TP, O’Brien NM (2004) Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia nut. Int J Food Sci Nutr 55:171–178
24. Bolling BW, McKay DL, Blumberg JB (2010) The phytochemical composition and antioxidant actions of tree nuts. Asia Pac J Clin Nutr 19:117–123
25. Amaral JS, Casal S, Pereira JA, Seabra RM, Oliveira BP (2003) Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (Juglans regia L.) cultivars grown in Portugal. J Agric Food Chem 51:7698–7702
26. Tapsell LC, Gillen LJ, Patch CS, Batterham M, Owen A, Bare M, Kennedy M (2004) Including walnuts in a low-fat/modified-fat diet improves HDL cholesterol-to-total cholesterol ratios in patients with type 2 diabetes. Diabetes Care 27:2777–2783
27. Iwamoto M, Sato M, Kono M, Hirooka Y, Sakai K, Takeshita A, Imaizumi K (2000) Walnuts lower serum cholesterol in Japanese men and women. J Nutr 130:171–176
28. Wu H, Pan A, Yu Z, Qi Q, Lu L, Zhang G, Yu D, Zong G, Zhou Y, Chen X, Tang L, Feng Y, Zhou H, Chen X, Li H, Demark-Wahnefried W, Hu FB, Lin X (2010) Lifestyle counseling and supplementation with flaxseed or walnuts influence the management of metabolic syndrome. J Nutr 140:1937–1942
29. Shimoda H, Tanaka J, Kikuchi M, Fukuda T, Ito H, Hatano T, Yoshida T (2008) Walnut polyphenols prevent liver damage induced by carbon tetrachloride and d-galactosamine: hepatoprotective hydroxylating tannins in the kernel pellicles of walnut. J Agric Food Chem 56:4444–4449
30. EN ISO 12228 (1999) Animal and vegetable fats and oils—determination of individual and total sterols contents—gas chromatographic method
31. Skrivanova E, Marounek M, De SS, Raes K (2007) Influence of dietary selenium and vitamin E on quality of veal. Meat Sci 76:495–500
32. Han A, Radin NS (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 90:420–426
33. Pinheiro J, Bates D, DebRoy S, Deepayan S, R Core Team (2012) nlme: linear and nonlinear mixed effects models. R package version 3.1-106
34. Fan JG, Qiao L (2009) Commonly used animal models of non-alcoholic steatohepatitis. Hepatobiliary Pancreat Dis Int 8:233–240
35. Hobbard L, George J (2011) Animal models of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 8:35–44
36. Ansee QM, Goldin RD (2006) Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol 87:1–16
37. Takahashi Y, Soejima Y, Fukusato T (2012) Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol 18:2300–2308
38. Liu Y, Meyer C, Xu C, Weng H, Hellerbrand C, Ten DP, Dooley S (2013) Animal models of chronic liver diseases. Am J Physiol Gastrointest Liver Physiol 304:G449–G468
39. Levy JR, Clore JN, Stevens W (2004) Dietary n-3 polysaturated fatty acids decrease hepatic triglycerides in Fischer 344 rats. Hepatology 39:608–616
40. Oosterveer MH, van Dijk TH, Tietje UJ, Boer T,Havinga R, Stellarda F, Groen AK, Kuipers F, Reijngoud DJ (2009) High fat feeding induces hepatic fatty acid elongation in mice. PLoS One 4:e6066–e6075
41. Yang ZH, Miyahara H, Takeo J, Hatanaka A, Katayama M (2003) Pollock oil supplementation modulates hyperlipidemia and ameliorates hepatic steatosis in mice fed a high-fat diet. Lipids Health Dis 10:189–198
42. Abete I, Goyenechea E, Zulet MA, Martinez JA (2011) Obesity and metabolic syndrome: potential benefit from specific nutritional components. Nutr Metab Cardiovasc Dis 21(Suppl 2):B1–B15
43. Fan JG, Qiao L (2009) Commonly used animal models of non-alcoholic steatohepatitis. Hepatobiliary Pancreat Dis Int 8:233-240
44. Hobbard L, George J (2011) Animal models of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 8:35-44
45. Ansee QM, Goldin RD (2006) Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol 87:1-16
46. Takahashi Y, Soejima Y, Fukusato T (2012) Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol 18:2300-2308
47. Liu Y, Meyer C, Xu C, Weng H, Hellerbrand C, Ten DP, Dooley S (2013) Animal models of chronic liver diseases. Am J Physiol Gastrointest Liver Physiol 304:G449-G468
48. Levy JR, Clore JN, Stevens W (2004) Dietary n-3 polysaturated fatty acids decrease hepatic triglycerides in Fischer 344 rats. Hepatology 39:608-616
49. Oosterveer MH, van Dijk TH, Tietje UJ, Boer T, Havinga R, Stellarda F, Groen AK, Kuipers F, Reijngoud DJ (2009) High fat feeding induces hepatic fatty acid elongation in mice. PLoS One 4:e6066-e6075
50. Yang ZH, Miyahara H, Takeo J, Hatanaka A, Katayama M (2003) Pollock oil supplementation modulates hyperlipidemia and ameliorates hepatic steatosis in mice fed a high-fat diet. Lipids Health Dis 10:189-198
51. Abete I, Goyenechea E, Zulet MA, Martinez JA (2011) Obesity and metabolic syndrome: potential benefit from specific nutritional components. Nutr Metab Cardiovasc Dis 21(Suppl 2):B1-B15
52. Lamaziere A, Wolf C, Barbe U, Bausero P, Visioli F (2013) Lipidomics of hepatic lipogenesis inhibition by omega 3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 88:149-154
53. Nergiz-Unal R, Kuipers MJ, de Witt SM, Heememan S, Feijige MA, Garcia Caraballo SC, Biessen EA, Haenen GR, Cosemans JM, Heemskerk JW (2013) Atheroprotective effect of dietary walnut intake in ApoE-deficient mice: involvement of lipids and coagulation factors. Thromb Res 131:411-417
54. MensenKamp AR, Jong MC, van Goor H, van Luyn MJ, Bloks V, Havinga R, Voshol PJ, Hofker MH, van Dijk KW, Havekes LM, Kuipers F (1999) Apolipoprotein E participates in the regulation of very low density lipoprotein-triglyceride secretion by the liver. J Biol Chem 274:35711-35718
56. Kuipers F, Jong MC, Lin Y, Eck M, Havinga R, Bloks V, Verkade HJ, Hofker MH, Moshage H, Berkel TJ, Vonk RJ, Havekes LM (1997) Impaired secretion of very low density lipoprotein-triglycerides by apolipoprotein E-deficient mouse hepatocytes. J Clin Invest 100:2915–2922

57. Masterton GS, Plevis RN, Hayes PC (2010) Review article: omega-3 fatty acids—a promising novel therapy for non-alcoholic fatty liver disease. Aliment Pharmacol Ther 31:679–692

58. Hussein N, Ah-Sing E, Wilkinson P, Leach C, Griffin BA, Millward DJ (2005) Long-chain conversion of 13C-linoleic acid and alpha-linolenic acid in response to marked changes in their dietary intake in men. J Lipid Res 46:269–280

59. Burdge GC, Wootton SA (2002) Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr 88:411–420

60. Harris WS (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res 30:785–807

61. Wong SH, Nestel PJ, Trimble RP, Storer GB, Illman RJ, Topping DL (1984) The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver. Biochem Biophys Acta 792:103–109

62. Harris WS, Bulchandani D (2006) Why do omega-3 fatty acids lower serum triglycerides? Curr Opin Lipidol 17:387–393

63. Brown AM, Wiggins D, Gibbons GF (1999) Glucose phosphorylation is essential for the turnover of neutral lipid and the second stage assembly of triacylglycerol-rich ApoB-containing lipoproteins in primary hepatocyte cultures. Arterioscler Thromb Vasc Biol 19:321–329

64. Ancal M, Pigna G, Favocchia C (2012) Mechanisms of diabetic dyslipidemia: relevance for atherogenesis. Curr Vasc Pharmacol 10:684–686

65. Podrini C, Borghesan M, Greco A, Pietanza V, Mazzoccoli G, Vaccari D, Torelli P, Torres D (2012) Redox homeostasis and epigenetics in non-alcoholic fatty liver disease (NAFLD). Curr Pharm Des 19:2737–2746

66. Botham KM, Zheng X, Napolitano M, Avella C, Cavallari C, Arca M, Pigna G, Favocchia C (2012) Dietary intake in men. J Biol Chem 278:604–607

67. Sampath H, Ntambi JM (2005) Polyunsaturated fatty acid regulation of genes regulating synthesis and secretion of very-low-density lipoprotein by the liver: modulation by cellular oxidative stress. Exp Biol Med (Maywood) 228:143–151

68. Torabian S, Haddad E, Cordero-MacIntyre Z, Tanzman J, Fernandez ML, Sabate J (2010) Long-term almond supplementation without dietary advice induces favorable serum lipid changes in free-living individuals. Eur J Clin Nutr 64:274–279

69. Davis PA, Vasu VT, Gohil K, Kim H, Khan IH, Cross CE, Yokoyama W (2012) A high-fat diet containing whole walnuts (Juglans regia) reduces tumour size and growth along with plasma insulin-like growth factor 1 in the transgenic adenocarcinoma of the mouse prostate model. Br J Nutr 108:1764–1772

70. Iwamoto M, Kono M, Kawamoto D, Tomoyori H, Sato M, Imaiizumi K (2002) Differential effect of walnut oil and safflower oil on the serum cholesterol level and lesion area in the aortic root of apolipoprotein E-deficient mice. Biosci Biotechnol Biochem 66:141–146

71. Sampath H, Ntambi JM (2005) Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu Rev Nutr 25:317–340

72. Wu T, Fu J, Yang Y, Zhang L, Han J (2009) The effects of phytosterols/stanols on blood lipid profiles: a systematic review with meta-analysis. Asia Pac J Clin Nutr 18:179–186

73. Kim HJ, Kim JH, Lee JW (1998) Steroid receptor coactivator-1 interacts with serum response factor and coactivates serum response element-mediated transactivations. J Biol Chem 273:28564–28567

74. Thakur ML, Sharma S, Kumar A, Bhatt SP, Luthra K, Guleria R, Pandey RM, Vikram NK (2012) Nonalcoholic fatty liver disease is associated with subclinical atherosclerosis independent of obesity and metabolic syndrome in Asian Indians. Atherosclerosis 223:507–511

75. Sookoian S, Castano GO, Burgueno AL, Rosselli MS, Gianotti V, Mallardi P, Martino JS, Pirola CJ (2010) Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. Atherosclerosis 209:585–591

76. Ito S, Yukawa T, Uetake S, Yamauchi M (2007) Steroid receptor coactivator-1 mediates hepatic steatosis and oxidative stress in obese Zucker (fa/ fa) rats. J Nutr 142:1956–1963

77. Raabe M, Veniant MM, Sullivan MA, Zlot CH, Bjorkgren J, Nielsen LB, Wong JS, Hamilton RL, Young SG (1999) Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. J Clin Invest 103:1287–1298

78. Chen Z, Newberry EP, Norris JY, Xie Y, Luo J, Kennedy SM, Davidson NO (2008) ApoB100 is required for increased VLDL-triglyceride secretion by microsomal triglyceride transfer protein in ob/ob mice. J Lipid Res 49:2013–2022

79. Banel DK, Hu FB (2009) Effects of walnut consumption on blood lipids and other cardiovascular risk factors: a meta-analysis and systematic review. Am J Clin Nutr 90:56–63

80. Torabian S, Haddad E, Cordero-MacIntyre Z, Tanzman J, Fernandez ML, Sabate J (2010) Long-term walnut supplementation without dietary advice induces favorable serum lipid changes in free-living individuals. Eur J Clin Nutr 64:274–279

81. Davis PA, Vasu VT, Gohil K, Kim H, Khan IH, Cross CE, Yokoyama W (2012) A high-fat diet containing whole walnuts (Juglans regia) reduces tumour size and growth along with plasma insulin-like growth factor 1 in the transgenic adenocarcinoma of the mouse prostate model. Br J Nutr 108:1764–1772

82. Iwamoto M, Kono M, Kawamoto D, Tomoyori H, Sato M, Imaiizumi K (2002) Differential effect of walnut oil and safflower oil on the serum cholesterol level and lesion area in the aortic root of apolipoprotein E-deficient mice. Biosci Biotechnol Biochem 66:141–146

83. Sampath H, Ntambi JM (2005) Polyunsaturated fatty acid regulation of genes of lipid metabolism. Annu Rev Nutr 25:317–340

84. Wu T, Fu J, Yang Y, Zhang L, Han J (2009) The effects of phytosterols/stanols on blood lipid profiles: a systematic review with meta-analysis. Asia Pac J Clin Nutr 18:179–186

85. Kim HJ, Kim JH, Lee JW (1998) Steroid receptor coactivator-1 interacts with serum response factor and coactivates serum response element-mediated transactivations. J Biol Chem 273:28564–28567

86. Thakur ML, Sharma S, Kumar A, Bhatt SP, Luthra K, Guleria R, Pandey RM, Vikram NK (2012) Nonalcoholic fatty liver disease is associated with subclinical atherosclerosis independent of obesity and metabolic syndrome in Asian Indians. Atherosclerosis 223:507–511

87. Sookoian S, Castano GO, Burgueno AL, Rosselli MS, Gianotti V, Mallardi P, Martino JS, Pirola CJ (2010) Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. Atherosclerosis 209:585–591

88. Ito S, Yukawa T, Uetake S, Yamauchi M (2007) Steroid receptor coactivator-1 mediates hepatic steatosis and oxidative stress in obese Zucker (fa/ fa) rats. J Nutr 142:1956–1963

89. Musso G, Anty R, Petta S (2013) Antioxidant therapy and drugs interfering with lipid metabolism: could they be effective in NAFLD patients? Curr Pharm Des 19:5297–5313

90. Yachi R, Igarashi O, Kiyose C (2010) Protective effects of dietary intervention in men. J Biol Chem 273:28564–28567

91. Thakur ML, Sharma S, Kumar A, Bhatt SP, Luthra K, Guleria R, Pandey RM, Vikram NK (2012) Nonalcoholic fatty liver disease is associated with subclinical atherosclerosis independent of obesity and metabolic syndrome in Asian Indians. Atherosclerosis 223:507–511

92. Ito S, Yukawa T, Uetake S, Yamauchi M (2007) Serum inter- cellular adhesion molecule-1 is elevated in nonalcoholic steatohepatitis: comparison with alcoholic hepatitis. Alcohol Clin Exp Res 31:S83–S87

93. Yachi R, Igarashi O, Kiyose C (2010) Protective effects of vitamin E analogs against carbon tetrachloride-induced fatty liver in rats. J Clin Biochem Nutr 47:148–154

94. Jacobs DR, Tapsell LC (2013) Food synergy: the key to a healthy diet. Proc Nutr Soc 72:200–206

95. Musso G, Anty R, Petta S (2013) Antioxidant therapy and drugs interfering with lipid metabolism: could they be effective in NAFLD patients? Curr Pharm Des 19:5297–5313