# Chronic Stress Potentiates High Fructose–Induced Lipogenesis in Rat Liver and Kidney

*Danijela Vojnović Milutinović, Jelena Brkljačić, Ana Teofilović, Biljana Bursać,
Marina Nikolić, Ljupka Gligorovska, Sanja Kovačević, Ana Djordjevic, Frederic Preitner,
Luc Tappy, Gordana Matić, and Nataša Veličković\**

**Scope:** Intake of fructose-sweetened beverages and chronic stress (CS) both increase risk of cardiometabolic diseases. The aim is to investigate whether these factors synergistically perturb lipid metabolism in rat liver and kidney. **Methods and results:** Fractional de novo lipogenesis (fDNL), intrahepatic- and intrarenal-triglycerides (IHTG and IRTG), de novo palmitate (DNPalm) content, FA composition, VLDL-TGs kinetics, and key metabolic gene expression at the end of the feeding and non-feeding phases in rats exposed to standard chow diet, chow diet + CS, 20% liquid high-fructose supplementation (HFr), or HFr+CS are measured. HFr induces hypertriglyceridemia, up-regulates fructose-metabolism and gluconeogenic enzymes, increases IHTG and DNPalm content in IHTG and IRTG, and augments fDNL at the end of the feeding phase. These changes are diminished after the non-feeding phase. CS does not exert such effects, but when combined with HFr, it reduces IHTG and visceral adiposity, enhances lipogenic gene expression and fDNL, and increases VLDL-DNPalm secretion. **Conclusion:** Liquid high-fructose supplementation increases IHTG and VLDL-TG secretion after the feeding phase, the latter being the result of stimulated hepatic and renal DNL. Chronic stress potentiates the effects of high fructose on fDNL and export of newly synthesized VLDL-TGs, and decreases fructose-induced intrahepatic TG accumulation after the feeding phase.

Dr. D. V. Milutinović, Dr. J. Brkljačić, Dr. A. Teofilović, Dr. B. Bursać,
Dr. M. Nikolić, L. Gligorovska, Dr. S. Kovačević, Dr. A. Djordjevic,
Prof. G. Matić, Dr. N. Veličković
Department of Biochemistry
Institute for Biological Research "Siniša Stanković"-National Institute of
Republic of Serbia
University of Belgrade
142 Despot Stefan Blvd., Belgrade 11000, Serbia
E-mail: nvelickovic@ibiss.bg.ac.rs
Dr. F. Preitner
Mouse Metabolic Facility (MEF), Center for Integrative Genomics
University of Lausanne
Lausanne CH-1015, Switzerland
Prof. L. Tappy
Department of Physiology
University of Lausanne, UNIL-CHUV
Rue du Bugnon 7, Lausanne CH-1005, Switzerland

## 1. Introduction

Consumption of a high-fructose diet may induce features of metabolic syndrome, including insulin resistance, dyslipidemia, and nonalcoholic fatty liver disease (NAFLD).[1] One of the proposed underlying mechanisms is increased hepatic de novo lipogenesis (DNL) that leads to increased atherogenic triglyceride-rich lipoprotein particles in the blood and ectopic lipid deposition in the liver and peripheral tissues.[2]

The liver is generally considered to be responsible for the disposal of the major portion of ingested fructose load. However, about 15% of ingested fructose may escape first-pass splanchnic metabolism, to be possibly metabolized by the kidney, adipose tissue, or other peripheral organs and tissues.[3] Therefore, fructose ingestion may also be a risk factor for chronic kidney disease due to possible fructose-induced disturbances of renal lipid metabolism.[4,5] A growing body of evidence also demonstrates a liver–kidney interaction in development of metabolic syndrome and NAFLD.[6] Fructose is similarly metabolized in both liver and kidney cells. It is rapidly phosphorylated by ketohexokinase C (KHK or fructokinase) into fructose 1-phosphate, which is subsequently cleaved by aldolase B into triose phosphates, substrates for gluconeogenesis, lipogenesis, or cellular respiration. Fructose especially provides a rapid substrate flux for DNL through uninhibited action of KHK and aldolase B that result in unregulated source of acetyl coenzyme A (acetyl-CoA). The main regulators of DNL are sterol regulatory element binding proteins (SREBPs), which act through transcriptional regulation of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD1) genes.[7] De novo synthesized fatty acids (FAs) can be either incorporated into intrahepatic/intrarenal or VLDL-triglycerides (IHTG, IRTG, VLDL-TGs, respectively) or oxidized to produce energy. The fraction of FAs that derives from DNL and is incorporated into VLDL-TGs is referred to as fractional DNL (fDNL). Products of fructose metabolism can also be directed toward gluconeogenesis, by the action of gluconeogenic genes

**ADVANCED
SCIENCE NEWS**

www.advancedsciencenews.com

**Molecular Nutrition
Food Research**

www.mnf-journal.com

phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase).[8]

Recent studies have demonstrated a correlation between exposure to chronic stress (CS) and increased cardiometabolic risk.[9] Stress-induced physiological and neuroendocrinological changes are associated with release of glucocorticoids, which have been related to weight gain, visceral fat deposition, alterations of hepatic and whole body glucose metabolism, and insulin resistance.[10] In addition, glucocorticoids can directly affect hepatic TGs secretion thereby contributing to hypertriglyceridemia, or can stimulate lipid accumulation by increasing the expression of genes involved in DNL.[11,12]

Interactions between chronic stress exposure and fructose overconsumption, as highly prevalent modern lifestyle characteristics, may play an important role in global epidemic of obesity and cardiometabolic diseases. Evidently, stimulated DNL is located in the hub of fructose and stress-induced metabolic disturbances. Therefore, the aim of this study was to examine the effects of liquid high-fructose supplementation (HFr) and chronic stress and their interaction on fDNL, de novo synthesized palmitate (DNPalm) content, IHTG and IRTG stores, hepatic and renal FA composition, VLDL-TGs kinetics, as well as on hepatic and renal gluconeogenic and lipogenic gene and protein expression profiles in male rats.

## 2. Experimental Section

### 2.1. Animals and Treatment

Male Wistar rats bred internal in the Animal facility of Institute for biological research "Siniša Stanković", aged 2.5 months at the beginning of the experiment, were randomly divided into four experimental groups ($n = 24$ animals per group). All animals had free access to standard chow diet (commercial food—Veterinary Institute, Subotica, Serbia)[13] until the end of treatments and were housed three per cage. The commercial food used in this study contained all necessary components in accordance with AIN-93 recommendations.[14] The experimental treatments were as follows: untreated control (C) group had free access to drinking water; HFr group received a 20% fructose (API-PEK, Bečej, Serbia) solution instead of drinking water during 9 weeks; CS group had free access to drinking water and was subjected to chronic unpredictable stress during the last 4 weeks of the treatment; HFr+CS group was exposed to both 9-week high-fructose supplementation and 4-week stress. The stress protocol was a modified protocol of Joels et al.[15] and included the following daily stressors: forced swimming in cold water for 10 min, physical restraint for 30 min, exposure to a cold room (4 °C) for 50 min, wet bedding for 4 h, rocking cages for 1 h, and cage tilt (45°) overnight. The number (1 or 2) and type of daily stressor(s), as well as the onset of stress exposure (between 9 AM and 4 PM for all the stressors) were randomly selected at the beginning of the treatment. A particular stressor was never applied on 2 consecutive days or twice per day. Food and fluid intakes were measured daily, while body mass was recorded weekly. Total energy intake was expressed as kJ per day per animal and was calculated as previously reported.[16] Animals were maintained under a 12 h light–dark cycle (lights on at 7 AM) at 22 °C and constant
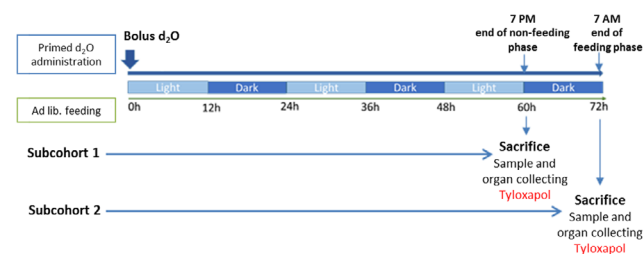


**Figure 1.** Graphical presentation of $D_2O$-labeling protocol and timeline of the experiments.

humidity. The protocols were performed in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković," University of Belgrade (reference number 02–11/14).

### 2.2. Deuterated Water-Labeling Protocol

Three days (72 h) before the end of the 9-week treatment period, all animals were subjected to deuterated water ($d_2O$)-labeling protocol as described by Turner et al.[17] Briefly, at 7 AM an initial priming dose of 99.8% $d_2O$ (Sigma Aldrich, USA) was injected intraperitoneally to achieve 6% body water enrichment (assuming that water constitutes 60% of body weight). Animals were then maintained on water or 20% fructose solution, both containing 6% $d_2O$, until the end of the treatment and were sacrificed at the end of either the feeding (7 AM) or non-feeding (7 PM) phases without being subjected to a fasting period. Actual deuterium enrichment in body water achieved throughout the study was 4.6%. The animals were further divided into subgroups for measurement of fDNL and VLDL-TG production rate.

### 2.3. Dosage Information

The dose of fructose (20% in the drinking water, ad libitum) was chosen in pilot experiment in order to allow the measurement of substrate fluxes with isotopic methods. The dose of $d_2O$ in both the priming injection and in the drinking water was chosen according to previous study.[18]

### 2.4. Measurement of fDNL

For fDNL measurements, animals from all groups subjected to $d_2O$-labeling protocol were divided in two subcohorts ($n = 6$ per experimental group): the first subcohort was sacrificed 60 h after the initial $d_2O$ injection at 7 PM, which corresponds to the end of the rest/non-feeding phase, while the second subcohort was sacrificed 72 h after the initial $d_2O$ injection at 7 AM, which corresponds to the end of the active/feeding phase (**Figure 1**). Rats are nocturnal animals that mostly consume food during night, which is therefore termed active or feeding phase. During the

day they rest, sleep, and generally do not consume food, so that this is referred to as rest or non-feeding phase.[19]

All animals were sacrificed by decapitation, trunk blood was rapidly collected into EDTA-containing tubes, and plasma was prepared by low-speed centrifugation ($1600 \times g$, 10 min, 4 °C). The livers were perfused with cold 0.9% NaCl, and the visceral adipose tissue (VAT), liver, and kidneys were carefully excised, weighted, and frozen in liquid nitrogen for further RNA, protein, and lipid isolation.

Hepatic and renal lipids were extracted by a modified Folch method using methyl-*tert*-butyl ether.[20] The total lipids in the organic phase were stored at −70 °C. For liver and kidney TGs measurement, the organic phase was dried under nitrogen stream, dissolved in chloroform/5% Triton X-100, dried again, and dissolved in 500 μL of ultrapure water.

For mass isotope analysis, the organic phase was dried under nitrogen stream and dissolved in chloroform containing 0.01% butylated hydroxytoluene. TGs were separated from other lipid fractions by SPE column (Interchim, 100 mg mL⁻¹), dried under nitrogen stream, resuspended in chloroform, and transmethylated with 3 N methanolic hydrochloric acid at 60 °C for 2 h. When cooled, 5% NaCl and hexane were added consecutively. After centrifugation ($863 \times g$, 10 min, 14 °C), the upper phase was evaporated under nitrogen stream and extracted FAMEs were dissolved in hexane and stored at −20 °C until use.

## 2.5. VLDL-TG Production Rate and Clearance

For measurement of VLDL-TG production rate, rats from the aforementioned four groups (C, HFr, CS, and HFr+CS) were subjected to the same interventions, including $d_2$O-labeling protocol 72 h before the end of the 9-week treatment. To measure VLDL-TG production rate, rats were anesthetized by a ketamine/xylazine mixture (80 and 10 mg kg⁻¹, respectively, IP) and received a retro-orbital injection of 500 mg kg⁻¹ Triton WR1339 (Tyloxapol, Sigma-Aldrich, USA) to inhibit TG hydrolysis by lipoprotein lipase.[21] The TG production rate was determined by calculating the increase of plasma TG over time after tyloxapol treatment. These animals were also divided into two subcohorts ($n = 6$ per experimental group): first subcohort received Tyloxapol injection 60 h after initial $d_2$O injection (7 PM) and the second subcohort 72 h after initial $d_2$O injection (7 AM) (Figure 1). Blood samples were drawn from tail tip into EDTA-containing tubes (at 0, 15, 30, 60, 90, and 120 min after injection) and plasma was prepared by low-speed centrifugation ($1600 \times g$, 10 min, 4 °C). The TG levels were measured by commercial triglycerides reagent (Biosystem, Spain). The pilot experiment revealed that linear increment of plasma TG was observed between 0 min and 2 h; therefore, the time points 0 (T0) and 60 min (T60) were further used to determine the VLDL-TG production rate by Equation (1) and VLDL clearance by Equation (2).

$$\text{VLDL-TG production rate } (\text{mmol h}^{-1})$$
$$= \text{VLDL-TG}_{T60}\text{-VLDL-TG}_{T0} \times \text{Plasma volume/1h} \qquad (1)$$

$$\text{VLDL-TG clearance } (\text{mL min}^{-1})$$
$$= \text{VLDL-TG production rate/VLDL-TG}_{T0} \qquad (2)$$

Both parameters for VLDL-TG kinetics were measured at the end of non-feeding (7 PM) and feeding (7 AM) phases.

Plasma VLDL fractions (density 1.006 g mL⁻¹) were separated by ultracentrifugation ($180\,000 \times g$/ 17 h/4 °C) using a Ti50.3 rotor (Beckman). The total TG level in the plasma, as well as in VLDL fraction was measured by commercial Randox TGs kit (Randox Laboratories Ltd., UK). Furthermore, FAMEs from VLDL-TG were isolated as previously described, dissolved in hexane and stored at −20 °C until use.

## 2.6. GC-MS Analysis and Calculations

For the analysis of hepatic and renal lipid contents, as well as for VLDL-TG production rate, FAMEs were analyzed for isotope enrichment by GC-MS. Mass isotopomer abundances were analyzed by selected ion monitoring: mass-to-charge ratios ($m/z$) 270–272 ($M0$ to $M2$) for palmitate-methyl esters. The liver and kidney samples isolated from additional animals that were not treated with $d_2$O served as the unlabeled control samples ($n = 6$ per time point). An Agilent model 6890/5973 GCMS (Agilent Technologies, USA) fitted with HP-INNOWax pure polyethylene glycol phase capillary GC column was used. The $^2$H labeling of water from plasma samples or standards was determined following the exchange with acetone.[22] The fDNL in VLDL-TGs (fDNPalm-VLDL) was calculated by mass isotopomer distribution analysis (MIDA).[23]

The amount of de novo synthesized palmitate content in IHTGs (IHTG-DNPalm content) was calculated at 7 AM and 7 PM as follows:

$$\text{IHTG-DNPalm content (mmol)}$$
$$= \text{Total intrahepatic TG (mmol)}$$
$$\times \text{fDNPalm−IHTG (\%) /100} \times \text{hepatic palmitate (\%) /100}$$

The same calculations were performed for de novo synthesized palmitate content in IRTG (IRTG-DNPalm content).

The amount of de novo synthesized palmitate secreted with VLDL at 7 AM and 7 PM was calculated as:

$$\text{VLDL-DNPalm secretion } (\text{mmol h}^{-1})$$
$$= \text{VLDL-TG production rate } (\text{mmol h}^{-1})$$
$$\times \text{fDNPalm−VLDL (\%) /100} \times \text{VLDL-palmitate (\%) /100}$$

## 2.7. FAMEs Analysis and Quantitation of Hepatic, Renal, and VLDL-TG Fatty Acid Composition

The amount of particular FA is presented as a percentage of the total FAs. The analyzed saturated FAs are palmitate (16:0) and stearate (18:0), MUFAs are palmitoleate (16:1n-7) and oleate (18:1 n-9), and PUFA is linoleate (18:2 n-6). Particular FA ratios were used to estimate relative activities of the FA elongase (18:1 n-9 + 18:0/16:0), Δ9 desaturase (16:1n-7/16:0, SCD-16 and 18:1n-9/18:0, SCD-18), and total DNL (16:0/18:2n-6).

### 2.8. Determination of Biochemical Parameters

The concentrations of glucose (Cat. number GL8038), free fatty acids (FFAs) (Cat. number FA115), lactate (Cat. number LC 2389) (all obtained from Randox Laboratories Ltd., UK), and insulin (Cat. number 90 060, Crystal Chem, USA) were measured in the plasma samples by commercial kits, while plasma, liver, and kidney TGs were analyzed with triglycerides reagent (Code 12528, Biosystem, Spain). All measurements were performed on the semi-automatic biochemistry analyzer Rayto 1904-C (Rayto, China).

### 2.9. Western Blot Analysis

Preparation of cell extracts for Western blot analysis was done as previously published.[24] The used primary antibodies are listed in Table S1 (Supporting Information). Membranes were subsequently washed and incubated with anti-rabbit (NA934, 1:20000, GE Healthcare) or anti-mouse (ab97046, 1:30000, Abcam) HRP-conjugated secondary antibodies. The immunoreactive proteins were visualized by enhanced chemiluminiscent method and quantitative analysis was performed by ImageJ software (National Institutes of Health, USA).

### 2.10. RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA was isolated from liver and kidney using TRIzol Reagent (Thermo Fisher Scientific, USA) according to manufacturer's instructions. Prior to cDNA synthesis, DNA contamination was removed by DNAse I treatment (Thermo Fisher Scientific, USA). Reverse transcription was performed using a high capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's instructions. The cDNAs were stored at −70 °C until use.

Quantification of FAS, ACC, and PEPCK gene expression in the liver was performed by TaqMan real-time polymerase chain reaction (PCR). The following probe sets were used: ACC (Rn00573474_m1), FAS (Rn01463550_m1), and PEPCK (Rn01529014_m1), all obtained from Applied Biosystems Assay-on Demand Gene Expression Products. $\beta$-Actin (Rn00667869_m1*) was used as endogenous control for quantitative normalization of cDNA. Real-time PCR was performed using the QuantStudio Real-Time PCR Systems as previously described.[25] The expression of aldolase B, apolipoprotein B (ApoB-100), G6Pase, glucose-6-phosphate dehydrogenase (G6PDH), Glut5, KHK, lactate dehydrogenase A (LDH-A), microsomal triglyceride transfer protein (MTTP), and SCD1 genes was analyzed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and specific primers (Metabion, Germany) (Table S2, Supporting Information). $\beta$-actin was used as endogenous control. Real-time PCR reaction was performed using QuantStudio Real-Time PCR Systems (Applied Biosystems, USA). Relative quantification of gene expression was examined using comparative $2^{-\Delta\Delta Ct}$ method. The results were analyzed by QuantStudio Design and Analysis v1.3.1 (Applied Biosystems, USA) with a confidence level of 95% ($p \leq 0.05$).

### 2.11. Statistical Analysis

The data are given as means ±SEM. Two-way ANOVA was used to evaluate effects of HFr and CS, and their interaction on treated groups. When there was significant interaction between factors, inter-group differences were analyzed by post hoc Tukey test. For statistical analyses STATISTICA 8.0 software (StatSoft, Inc., USA) was used and statistical significance was accepted at $p < 0.05$.

## 3. Results

### 3.1. Food and Energy Intake, Body Composition, and Biochemical Parameters

Food intake was decreased ($F_{1,39} = 98.6$, $p < 0.001$) while total energy intake ($F_{1,39} = 12.3$, $p < 0.01$) was increased by HFr (**Table 1**). Concomitantly, HFr led to alteration in body composition, as revealed by increased VAT mass ($F_{1,39} = 9.6$, $p < 0.001$) and VAT-to-body ratio ($F_{1,39} = 12.4$, $p < 0.01$, Table 1). CS reduced VAT mass ($F_{1,39} = 11.4$, $p < 0.01$) and VAT-to-body ratio ($F_{1,39} = 6.8$, $p < 0.05$) in the HFr+CS group (Table 1). Neither CS nor HFr had significant effects on liver and kidney masses or on their ratio to body mass (Table 1).

HFr led to increased plasma TG and glucose after both feeding ($F_{1,15} = 188.9$, $p < 0.001$, for TG; $F_{1,15} = 24.4$, $p < 0.001$, for glucose) and non-feeding phases ($F_{1,17} = 20.4$, $p < 0.001$ for TG; $F_{1,17} = 6.1$, $p < 0.05$ for glucose), while plasma lactate and insulin were not affected (Table 2). VLDL-TG production rate was not altered, but VLDL-TG clearance was markedly decreased by HFr after both feeding ($F_{1,17} = 1.7$, $p < 0.01$) and non-feeding ($F_{1,17} = 5.5$, $p < 0.05$) phases, which resulted in elevated plasma VLDL-TG concentration after both phases ($F_{1,17} = 13.4$, $p < 0.01$; $F_{1,17} = 7.2$, $p < 0.05$, respectively) (Table 2). CS alone had no significant effect on these parameters, except for increase of blood glucose ($F_{1,17} = 5.6$, $p < 0.05$), and a slight decrease in plasma FFA ($F_{1,17} = 6.7$, $p < 0.05$) and lactate ($F_{1,17} = 11.0$, $p < 0.01$) concentration at the end of feeding phase (Table 2). In HFr +CS, CS enhanced HFr-induced increment of plasma VLDL-TG concentration at the end of the feeding phase (CS: $F_{1,17} = 5.3$, $p < 0.05$, Table 2). An interaction of HFr and CS was evident for plasma TG concentration after both phases (HFr x CS: $F_{1,15} = 39.3$, $p < 0.001$; $F_{1,17} = 5.7$, $p < 0.05$, respectively) and for plasma glucose only after the feeding phase (HFr x CS: $F_{1,15} = 11.0$, $p < 0.01$) (Table 2).

### 3.2. Hepatic and Renal Fructose Transporters and Fructose-Metabolizing and Gluconeogenic Enzymes

Since postprandial fructose is mainly metabolized in the liver and to some extent in the kidney, we analyzed fructose transporters and enzymes of fructolysis in these tissues. As expected, HFr provoked an increase in the fructose transporter Glut2 level in the liver ($F_{1,18} = 7.0$, $p < 0.05$) and expression of fructose-metabolizing enzyme KHK ($F_{1,18} = 15.9$, $p < 0.01$) (**Figure 2**a). On the other hand, gene expression of two main gluconeogenic enzymes in the liver, PEPCK, and G6Pase, was unaltered (Figure 2a). The expression of LDH-A gene, an enzyme responsible

**ADVANCED
SCIENCE NEWS**

www.advancedsciencenews.com

**Molecular Nutrition
Food Research**

www.mnf-journal.com

**Table 1.** The effects of high-fructose supplementation and chronic stress on body mass, tissue mass, and energy intake in male Wistar rats.

|  | C | HFr | CS | HFr+CS |
|---|---|---|---|---|
| Food intake (kJ day$^{-1}$ per animal) | 229.5± 7.2 | 136 ± 8.5$^A$ | 233.5 ± 16.1 | 132.5 ± 3.8$^A$ |
| Fructose intake (kJ day$^{-1}$ per animal) | / | 143.8 ± 9.3 | / | 132.3 ± 3.8 |
| Total energy intake (kJ day$^{-1}$ per animal) | 229.5± 7.2 | 279.8 ± 15.6$^A$ | 233.5 ± 16.1 | 264.8 ± 4.0$^A$ |
| Body mass (g) | 352.2± 13.6 | 360.6 ± 8.3 | 327.6 ± 7.1 | 337.2 ± 9.5 |
| VAT mass (g) | 6.92 ±0.72 | 9.47 ± 0.72$^A$ | 5.39 ± 0.40$^B$ | 6.74 ±0.6$^{A,B}$ |
| VAT/body ratio (×100) | 1.94± 0.16 | 2.63 ± 0.20$^A$ | 1.65 ± 0.11$^B$ | 2.09 ± 0.16$^{A,B}$ |
| Liver mass (g) | 14.54 ± 1.3 | 16.57 ± 1.97 | 13.75 ± 2.22 | 15.78 ± 1.27 |
| Liver/body ratio (×100) | 3.81 ± 0.31 | 4.02 ± 0.62 | 3.97 ± 0.46 | 3.60 ± 0.83 |
| Kidney mass (g) | 1.04 ± 0.03 | 1.04± 0.05 | 1.03 ± 0.04 | 0.9 ± 0.03 |
| Kidney/body ratio (× 1000) | 3.08 ± 0.04 | 2.92 ± 0.23 | 3.29 ± 0.15 | 3.00 ± 0.17 |

Energy obtained from food and fructose intake was measured twice per week and total energy intake was calculated per day and per animal at the end of the treatment (9 weeks on liquid high-fructose supplementation [HFr] combined with 4 weeks of chronic unpredictable stress [CS]). Visceral adipose tissue (VAT) mass, VAT-to-body ratio, liver and kidney masses, and their ratio to body weight were measured at the sacrifice. The data are presented as means ± SEM ($n$ = 11–12). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Different letters denote statistically significant main effect of HFr (A) or CS (B).

**Table 2.** The effects of high-fructose supplementation and chronic stress on biochemical parameters and VLDL-TG kinetics in male Wistar rats.

|  |  | C | HFr | CS | HFr+CS |
|---|---|---|---|---|---|
| TGs (mmol L$^{-1}$) | 7 AM | 0.56± 0.03 | 1.56 ± 0.09$^A$ | 0.82 ± 0.02 | 1.20 ± 0.04$^{***,\,\$\$\$,\#\#\#,}$ |
|  | 7 PM | 0.82 ± 0.07 | 1.12 ± 0.16$^A$ | 0.58 ± 0.05 | 1.56 ± 0.2$^{**,\#\#\#}$ |
| FFA (mmol L$^{-1}$) | 7 AM | 0.23 ± 0.04 | 0.39 ± 0.03$^A$ | 0.19 ± 0.03$^B$ | 0.24 ± 0.04$^{A,B}$ |
|  | 7 PM | 0.25 ± 0.04 | 0.30 ± 0.05 | 0.24 ± 0.04 | 0.32 ± 0.04 |
| Glucose (mmol L$^{-1}$) | 7 AM | 5.44 ± 0.30 | 7.42 ± 0.22$^A$ | 6.41 ± 0.15$^B$ | 6.80 ± 0.26$^{**}$ |
|  | 7 PM | 5.52 ± 0.19 | 8.33 ± 1.02$^A$ | 6.74 ± 0.88 | 7.24 ± 0.33$^A$ |
| Insulin (ng mL$^{-1}$) | 7 AM | 2.66 ± 0.29 | 3.70 ± 0.38 | 3.09 ± 0.33 | 2.85 ± 0.35 |
|  | 7 PM | 5.33 ± 1.04 | 4.00 ± 0.43 | 2.54 ± 0.59 | 3.74 ± 0.85 |
| Lactate (mmol L$^{-1}$) | 7 AM | 2.21 ± 0.09 | 2.46 ± 0.19 | 1.67 ± 0.10$^B$ | 1.99 ± 0.18$^B$ |
|  | 7 PM | 2.35 ± 0.17 | 2.15 ± 0.28 | 1.73 ± 0.18 | 1.99 ± 0.27 |
| Plasma VLDL-TG (mmol L$^{-1}$) | 7 AM | 0.59 ± 0.08 | 0.80 ± 0.06$^A$ | 0.67 ± 0.10$^B$ | 1.15 ± 0.11$^{A,B}$ |
|  | 7 PM | 0.52 ± 0.14 | 0.81 ± 0.14$^A$ | 0.58 ± 0.12 | 1.01 ± 0.13$^A$ |
| VLDL-TG production rate (mmol h$^{-1}$) | 7 AM | 2.01 ± 0.10 | 2.03± 0.17 | 1.76± 0.19 | 1.78± 0.15 |
|  | 7 PM | 2.35 ± 0.17 | 2.15± 0.28 | 1.73± 0.18 | 1.99± 0.27 |
| VLDL clearance (mL min$^{-1}$) | 7 AM | 0.67 ± 0.16 | 0.37 ± 0.10$^A$ | 0.65 ± 0.11 | 0.30 ± 0.06$^A$ |
|  | 7 PM | 0.65 ± 0.17 | 0.35 ± 0.06$^A$ | 0.52 ± 0.12 | 0.29 ± 0.07$^A$ |

Blood triglycerides (TGs) and glucose, plasma free fatty acids (FFA), insulin and lactate, plasma VLDL-TG, and VLDL-TG production rate and clearance were measured in the rats after 9 weeks of liquid high-fructose supplementation (HFr) and 4 weeks of exposure to chronic unpredictable stress (CS) after feeding (7 AM) and non-feeding phase (7 PM). The data are presented as means ± SEM ($n$ = 5–6). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Letters denote statistically significant main effect of HFr (A) or CS (B). Inter-group differences were analyzed with the post hoc Tukey test when significant interaction between factors was observed. Different symbols denote statistically significant difference between groups ($^{**}p < 0.01$, $^{***}p < 0.001$ vs C; $^{\$\$\$}p < 0.001$ vs HFr; $^{\#\#\#}p < 0.001$ vs CS).

for the reversible conversion of pyruvate to lactate in the liver, was increased by HFr ($F_{1,18} = 8.1$, $p < 0.05$), while the level of G6PDH, the enzyme generating NADPH, was not changed (Figure 2a). CS did not change the expression of the above mentioned enzymes, but combined HFr+CS induced elevation of aldolase B mRNA level in the liver (HFr × CS: $F_{1,18} = 6.22$, $p < 0.05$, Figure 2a).

In the kidney, protein level of the fructose transporter Glut2 was not changed (Figure S1, Supporting Information), while Glut5, KHK, and aldolase B mRNAs were all increased by HFr ($F_{1,20} = 56.7$, $p < 0.001$; $F_{1,20} = 24.0$, $p < 0.001$; $F_{1,20} = 66.8$, $p < 0.001$, respectively; Figure 2b). In contrast to the liver, the

levels of renal PEPCK and G6Pase mRNA were elevated by HFr ($F_{1,20} = 12.1$, $p < 0.01$; $F_{1,20} = 44.3$, $p < 0.001$, respectively). On the other hand, renal LDH-A mRNA level was not changed by any of the applied treatments, while HFr affected G6PDH mRNA in the kidney of the treated animals ($F_{1,20} = 12.9$, $p < 0.01$, Figure 2b).

### 3.3. Hepatic and Renal Lipid Metabolism

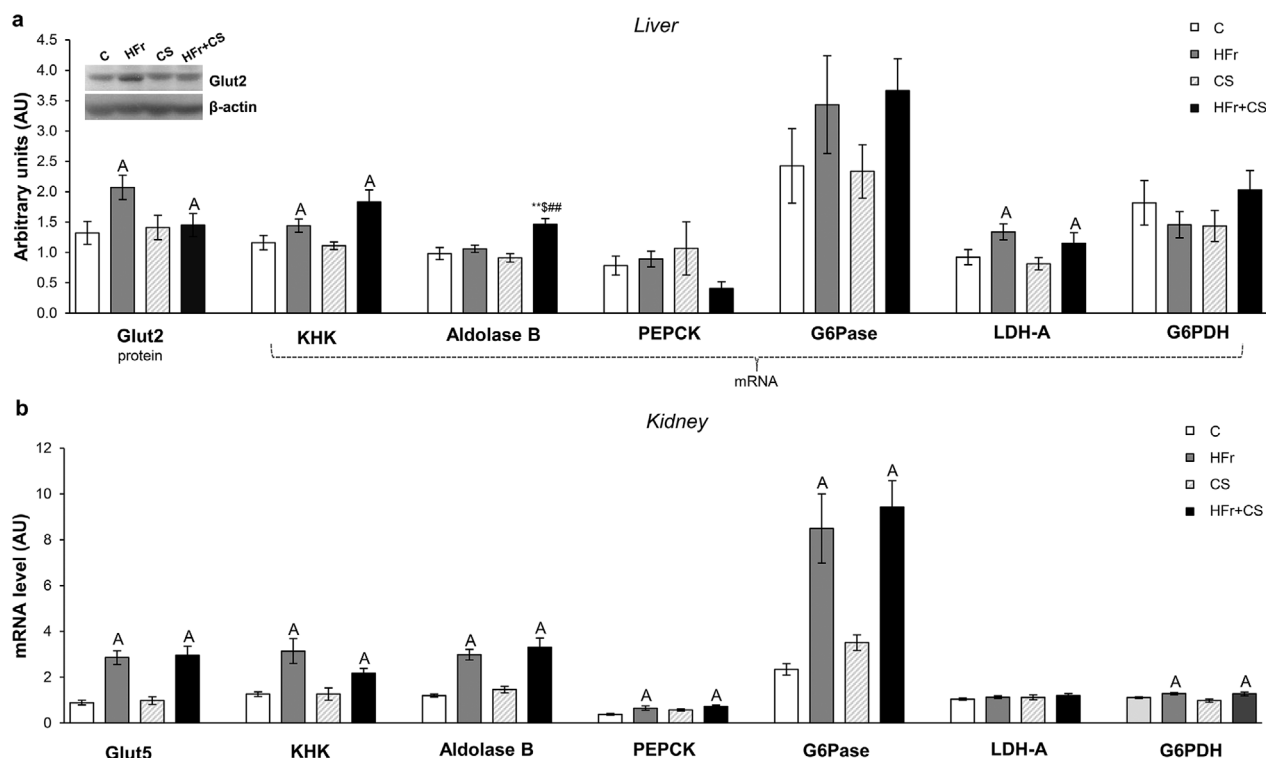The main consequence of fructose overconsumption is uncontrolled production of precursors for DNL and stimulated

**Figure 2.** Expression of fructose transporters, enzymes of fructolysis and gluconeogenesis, lactate dehydrogenase A (LDH-A), and glucose-6-phosphate dehydrogenase (G6PDH) in the liver and kidney. The level of Glut2 protein and mRNA for Glut5, ketohexokinase (KHK), aldolase B, glucose 6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), LDH-A, and G6PDH in the liver (a) and kidney (b) of control (C), liquid high-fructose supplementation (HFr), chronic stress (CS), and high-fructose + chronic stress (HFr+CS) groups after feeding phase (7 AM). Values are means ± SEM ($n = 5$–6 rats per group). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Letter A denotes statistically significant main effect of HFr. Inter-group differences were analyzed with the post hoc Tukey test when significant interaction between factors was observed. Different symbols denote statistically significant difference between groups ($^{**}p < 0.01$ vs C; $^{\$}p < 0.05$ vs HFr; $^{\#\#}p < 0.01$ vs CS).

de novo synthesis of FA. To assess fDNL, we performed $d_2O$-labeling protocol to measure label incorporation in palmitate, the main product of DNL. Importantly, the FA synthesis highly depends on feeding/non-feeding cycle, therefore, we performed measurements of de novo synthesized palmitate content (DNPalm content) in IHTG and IRTG and on VLDL-TGs kinetics at 7 AM (end of feeding phase) and 7 PM (end of non-feeding phase). HFr significantly increased IHTG after feeding phase ($F_{1,20} = 5.6$, $p < 0.05$), but significantly reduced it after non-feeding phase ($F_{1,17} = 6.4$, $p < 0.05$) (**Table 3**). IHTG-DNPalm content was also increased in HFr animals after feeding phase ($F_{1,17} = 17$, $p < 0.01$), but remained unchanged after the non-feeding phase (Table 3). Although HFr did not affect IRTG stores, it increased the IRTG-DNPalm content after the feeding ($F_{1,20} = 4.2$, $p < 0.05$), but not after the non-feeding phase (Table 3). To assess whether HFr also modulated lipoprotein TG secretion, we measured de novo synthesized palmitate secreted with VLDL (VLDL-DNPalm secretion). The animals on HFr diet displayed stimulated fDNL in the VLDL-TGs after the feeding phase (fDNL-VLDL) ($F_{1,18} = 29.2$, $p < 0.001$), and increased VLDL-DNPalm secretion after the same phase (HFr: $F_{1,16} = 7.4$, $p < 0.05$) (Table 3).

CS affected IHTG stores ($F_{1,20} = 6.5$, $p < 0.05$) and IRTG-DNPalm content ($F_{1,20} = 4.9$, $p < 0.05$), leading to reduced accumulation of IHTG, but not to change of IRTG (Table 3). In-

terestingly, CS additionally increased IRTG-DNPalm content in HFr+CS group after feeding phase (CS: $F_{1,20} = 6.3$, $p < 0.05$). As in the case of HFr animals, this was not observed after the non-feeding phase (Table 3). Combined HFr and CS enhanced fDNL-VLDL (HFr × CS: $F_{1,18} = 9.9$, $p < 0.01$), and the release of VLDL-DNPalm (HFr × CS: $F_{1,16} = 3.5$, $p = 0.08$) after the feeding phase (Table 3).

The synergistic effects of HFr and CS on fDNL dynamics in the examined tissues were in accordance with the expression levels of genes encoding enzymes and proteins involved in lipid metabolism and VLDL formation. The expression of hepatic lipogenic genes (ACC, FAS, and SCD1) and ApoB-100 gene was not changed by HFr diet nor by CS, except for MTTP mRNA that was increased after CS treatment (HFr × CS: $F_{1,16} = 7.5$, $p < 0.05$) (**Figure 3**a). However, the expression of ACC, FAS, and SCD1 genes was significantly increased in the liver of HFr+CS animals as a result of interaction between factors (HFr x CS: $F_{1,17} = 6.9$, $p < 0.05$, for ACC; $F_{1,17} = 8.9$, $p < 0.01$ for FAS; $F_{1,17} = 21.4$, $p < 0.001$ for SCD1) (Figure 3a). The induction of lipogenic genes in HFr+CS group was accompanied by a trend toward increased lipogenic transcription factor SREBP-1c, while the expression of ChREBP and LXR were not affected by any of the treatments (**Figure 4**a). Interestingly, the analysis of the levels of ACC protein and its form phosphorylated on Ser[79]

**Table 3.** The effects of high-fructose supplementation and chronic stress on fDNL in the liver, kidney and VLDL triglycerides (TGs).

| | | C | HFr | CS | HFr + CS |
|---|---|---|---|---|---|
| IHTG (mg g$^{-1}$) | 7 AM | 24.74 ± 4.25 | 47.40 ± 9.82[A] | 19.61 ± 3.99[B] | 23.73 ± 3.49[A,B] |
| | 7 PM | 56.68 ± 13.66 | 20.24 ± 1.84[A] | 31.10 ± 5.55 | 28.58 ± 5.24[A] |
| IHTG-DNpalm content (mmol) | 7 AM | 0.11 ± 0.04 | 0.51 ± 0.19[A] | 0.11 ± 0.06 | 0.38 ± 0.08 [A] |
| | 7 PM | 0.08 ± 0.02 | 0.07 ± 0.01 | 0.04 ± 0.02 | 0.091 ± 0.02 |
| IRTG (mg g$^{-1}$) | 7 AM | 4.11 ± 0.86 | 5.88 ± 0.78 | 7.82 ± 1.25 | 6.28 ± 0.94 |
| | 7 PM | 7.51 ± 1.81 | 10.39 ± 3.44 | 6.03 ± 2.38 | 7.60 ± 1.62 |
| IRTG-DNPalm content (μ mol) | 7 AM | 0.27 ± 0.10 | 1.08 ± 0.31 [A] | 1.21 ± 0.48[B] | 3.55 ± 1.67[A,B] |
| | 7 PM | 0.51 ± 0.19 | 1.61 ± 0.79 | 0.15 ± 0.09 | 1.1 ± 0.79 |
| fDNL-VLDL (%) | 7 AM | 44.9 ± 5.7 | 66.7 ± 8.4[A] | 30.9 ± 3.7 | 85.2 ± 8.5[**,###] |
| | 7 PM | 27.7 ± 3.8 | 40.3 ± 9.2 | 26.6 ± 6.1 | 37.7 ± 3.1 |
| VLDL-DNPalm secretion (mmol h$^{-1}$) × 100 | 7 AM | 0.31 ± 0.13 | 0.42 ± 0.02 [A] | 0.27 ± 0.06 | 0.84 ± 0.16[*,#] |
| | 7 PM | 0.15 ± 0.04 | 0.37 ± 0.17 | 0.20 ± 0.09 | 0.38 ± 0.07 |

Hepatic TGs (IHTG), renal TGs (IRTG), de novo palmitate content (fDNPalm content) in IHTG and IRTG, fractional de novo lipogenesis (fDNL) in VLDL, and fractional de novo VLDL-palmitate secretion (VLDL-fDNPalm secretion) were measured in the rats after 9 weeks on liquid high-fructose supplementation (HFr) and 4 weeks of exposure to chronic unpredictable stress (CS) after feeding (7 AM) and non-feeding phase (7 PM). The data are presented as means ± SEM ($n = 5$–6). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Different letters denote statistically significant main effects of HFr (A) or CS (B). Inter-group differences were analyzed by the post hoc Tukey test when significant interaction between factors was observed. Different symbols denote statistically significant differences between groups (*$p < 0.05$, **$p < 0.01$ vs C; ##$p < 0.05$ vs CS).
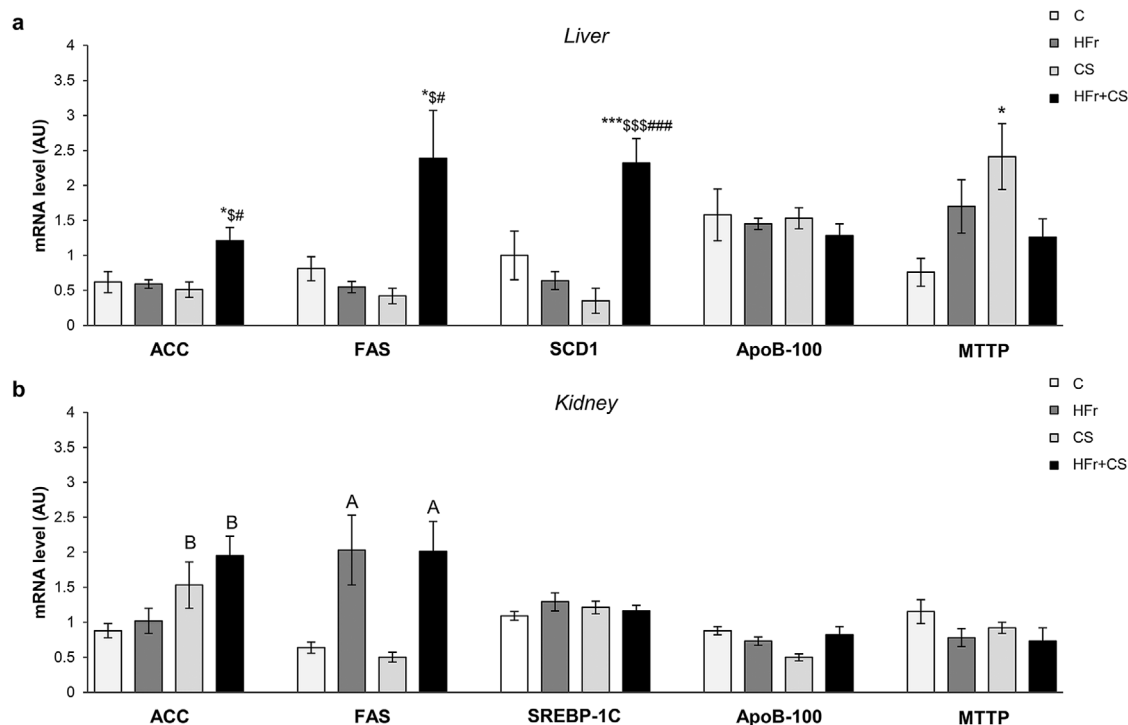


**Figure 3.** Expression of genes involved in lipid metabolism in the liver and kidney. The level of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), apolipoprotein B-100 (ApoB-100), SREBP-1c, and MTTP mRNAs relative to $\beta$-actin mRNA were measured in the liver (a) and kidney (b) of control (C), liquid high-fructose supplementation (HFr), chronic stress (CS), and high-fructose + chronic stress (HFr+CS) groups after feeding phase (7 AM). All data are presented as means ± SEM ($n = 5$–6). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Different letters denote statistically significant main effect of HFr (A) or CS (B). Inter-group differences were analyzed by the post hoc Tukey test when significant interaction between factors was observed. Different symbols denote statistically significant differences between groups ($^{*}p < 0.05$ versus C; $^{\$}p < 0.05$ versus HFr; $^{\#}p < 0.05$ versus CS).
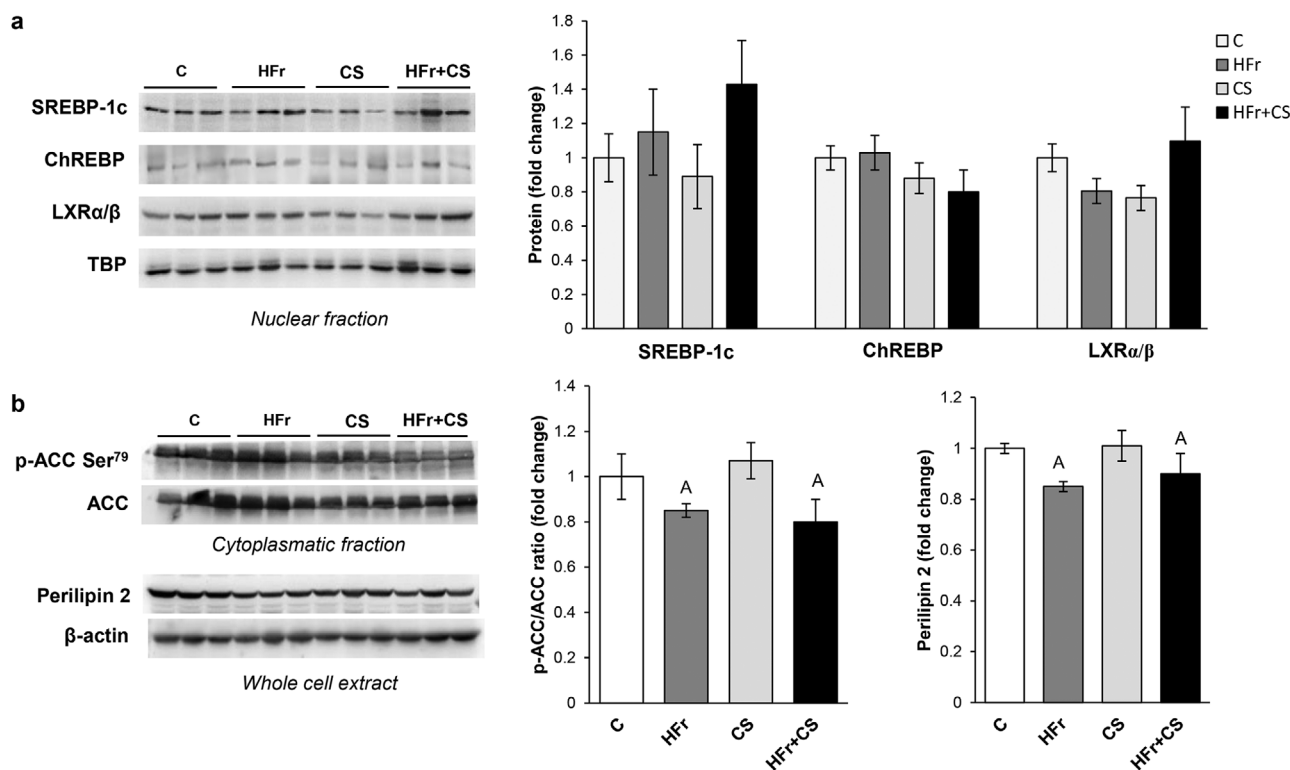
**Figure 4.** Expression of proteins involved in hepatic lipid metabolism. The level of lipogenic transcription factors SREBP-1c, ChREBP and LXR (a), phospho-ACC Ser[79]/ACC, and perilipin 2 (b) were measured in the liver of control (C), liquid high-fructose supplementation (HFr), chronic stress (CS), and high-fructose + chronic stress (HFr+CS) groups after feeding phase (7 AM). All data are presented as means ± SEM ($n = 6$). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Letter A denotes statistically significant main effect of HFr.

revealed that phospho-ACC/ACC ratio was decreased by HFr ($F_{1,19} = 8.9$, $p < 0.01$) (Figure 4b), which is in accordance with increased IHTG-DNPalm content (Table 3). In order to examine the direction of de novo synthesized lipid mobilization, the expression of perilipin 2, a crucial protein for hepatic lipid droplet formation, was examined. Results demonstrated that HFr significantly reduced perilipin 2 protein content ($F_{1,19} = 7.3$, $p < 0.05$) (Figure 4b). In the kidney, HFr significantly increased FAS mRNA ($F_{1,20} = 18.2$, $p < 0.01$), while CS affected renal ACC mRNA ($F_{1,20} = 10.6$, $p < 0.01$, Figure 3b). The expression of SREBP-1c, MTTP, and ApoB-100 genes was not changed in the kidney after the applied treatments (Figure 3b). After the food-restricted phase, the alterations in the expression of these genes were not observed in any group or tissue (data not shown).

### 3.4. FA Composition in the Liver and Kidney

Lipogenic indexes for FA elongation, desaturation, and total DNL were estimated from the changes in IHTG and IRTG FA composition. The elongation and Δ9 desaturase indexes, which correspond to activity of elongase, C16, and C18 desaturase enzymes, were increased as a result of main effect of HFr ($F_{1,15} = 41.7$, $p < 0.001$, for elongation index; $F_{1,14} = 16.5$, $p < 0.001$; $F_{1,14} = 9.1$, $p < 0.01$, for C16 and C19 desaturase index, respectively) (Table 4).

The DNL index represents the ratio of endogenously produced palmitic acid, the main product of DNL, and the essential linoleic acid originating from dietary lipids. HFr significantly increased DNL index ($F_{1,14} = 26.9$, $p < 0.001$), which is in accordance with stimulated fDNL in these animals (Table 4). In the CS group, none of these indexes were changed, while CS in combination with HFr led to significantly increased DNL index (CS: $F_{1,14} = 4.6$, $p < 0.05$).

In the kidney, HFr was the main factor that increased elongation index and Δ9 C18 desaturase index ($F_{1,16} = 14.7$, $p < 0.01$; $F_{1,16} = 14.2$, $p < 0.01$, respectively), while neither stress nor combined treatment affected these lipogenic indexes (Table 4).

In line with increased IHTG-DNPalm content and Δ9 C16 desaturase index, the palmitate and palmitoleate percentage in the total hepatic FAs were increased in HFr animals ($F_{1,16} = 21.6$, $p < 0.001$; $F_{1,15} = 42.3$, $p < 0.001$, respectively) (**Figure 5**a). Even though stearate was not changed, the oleate was significantly increased by HFr ($F_{1,15} = 42.8$, $p < 0.001$), probably as a result of stimulated desaturation. On the other hand, the linoleate was significantly decreased in HFr animals ($F_{1,15} = 44.9$, $p < 0.001$) (Figure 5a), which is in line with decreased food intake. In the kidney, only increased percentage of palmitate was observed in the HFr group ($F_{1,16} = 54.7$, $p < 0.001$) (Figure 5b), in accordance with increased DNPalm content in IRTG.

**Table 4.** The indexes of FA elongation, desaturation, and total DNL in the liver and kidney.

| Name and Index | C | HFr | CS | HFr +CS |
|---|---|---|---|---|
| Liver | | | | |
| Elongation (18:1 (n-9)+18:0)/16:0 | 10.19 ± 0.43 | 13.37 ± 0.43[A] | 9.95 ± 0.30 | 12.32 ± 0.54[A] |
| Δ9D C16 16:1 (n-7)/16:0 | 0.027 ± 0.008 | 0.065 ± 0.004[A] | 0.027 ± 0.003 | 0.063 ± 0.005[A] |
| Δ9D C18 18:1 (n-9)/18:0 | 1.59 ± 0.17 | 2.02 ± 0.21[A] | 1.29 ± 0.13 | 1.68 ± 0.13[A] |
| DNL 16:0/18:2 (n-6) | 1.41 ± 0.06 | 4.04 ± 0.45[A] | 1.83 ± 0.17[B] | 6.72 ± 0.54[A,B] |
| Kidney | | | | |
| Elongation (18:1 (n-9)+18:0)/16:0 | 1.69 ± 0.14 | 2.46 ± 0.20[A] | 1.71 ± 0.08 | 2.07 ± 0.14[A] |
| Δ9D C16 16:1 (n-7)/16:0 | 0.019 ± 0.003 | 0.027 ± 0.006 | 0.023 ± 0.003 | 0.028 ± 0.004 |
| Δ9D C18 18:1 (n-9)/18:0 | 0.34 ± 0.01 | 0.62 ± 0.07[A] | 0.43 ± 0.01 | 0.57 ± 0.08[A] |
| DNL 16:0/18:2 (n-6) | 3.12 ± 0.49 | 3.29 ± 0.28 | 2.84 ± 0.47 | 3.73 ± 0.33 |

The data are presented as means ± SEM ($n$ = 5–6). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Different letters denote statistically significant main effect of HFr (A) or CS (B). C, control; HFr, liquid high-fructose supplementation; CS, chronic stress; HFr+CS, high-fructose + chronic stress group.
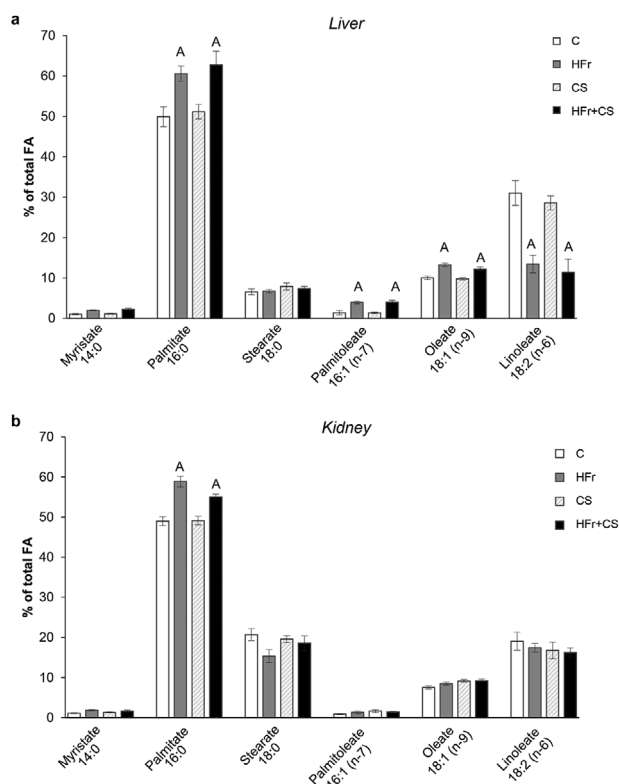


**Figure 5.** Fatty acid compositions in the liver (a) and kidney (b) after feeding phase (7 AM). The amount of particular FA is presented as a percentage of the total FAs level. All data are presented as means ± SEM ($n$ = 5–6). Two-way ANOVA was used to evaluate the effects of HFr and CS, and their interaction on treated groups. Letter A denotes statistically significant main effect of HFr. C, control; HFr, liquid high-fructose supplementation; CS, chronic stress; HFr+CS, high-fructose + chronic stress group.

## 4. Discussion

This study was aimed at assessing whether the effects of an HFr on plasma VLDL-TG and glucose concentrations may be synergized by concomitant CS. To reach this goal, we monitored how HFr, CS, and HFr+CS affect key metabolic processes regulating plasma VLDL-TG and glucose concentrations. Since these metabolic processes are dynamic and dependent on food intake patterns, we studied the effects of HFr at the end of both active (feeding) and rest (non-feeding) phases.

Many studies have linked fructose overconsumption and/or chronic stress with the development of obesity and visceral adiposity.[1,10] In the present study, HFr decreased solid food intake, but increased total energy intake as a result of fructose-ingested calories. This was not associated with a significant increase in body mass, liver, or kidney mass, but was related to an increased VAT and VAT-to-body ratio. CS did not change total energy intake nor body, liver, or kidney mass, but reduced VAT in the HFr+CS group, which was possibly the result of glucocorticoid-induced lipolysis.[26]

HFr significantly increased blood glucose at the end of the feeding phase, but it did not affect the plasma insulin. Such fructose-induced hyperglycemia has been well documented in previous fructose overfeeding studies, and has been essentially attributed to increased hepatic glucose output and impaired hepatic or systemic insulin sensitivity.[27] In this study, expression of gluconeogenic genes was not significantly altered by HFr, suggesting that increased hepatic glucose production was mainly stimulated at the substrate levels, that is, by increased intrahepatic triose phosphate concentrations. Nevertheless, impaired glucose tolerance, which is a hallmark of hepatic and systemic insulin resistance, and increased inhibitory phosphorylation of IRS1 in hepatocyte, which is a marker of hepatic insulin resistance, were previously observed in HFr animals.[28] Noteworthy, in this former study, hyperinsulinemia and hypoglycemia were observed in HFr animals, which is most likely a result of overnight fasting.[29] In the current study, HFr increased the expression of fructose transporters and fructolysis enzymes in both liver and kidney, as well as renal expression of gluconeogenic genes, indicating that whatever portion of fructose escaped first-pass splanchnic uptake, it was sufficient to up-regulate fructose and glucose metabolism in the kidney.

HFr expectedly induced hypertriglyceridemia and increased plasma VLDL-TG. This could be explained by a decreased VLDL clearance and reduced perilipin 2 protein expression, which has

**ADVANCED
SCIENCE NEWS**

www.advancedsciencenews.com

**Molecular Nutrition
Food Research**

www.mnf-journal.com

been shown to promote the secretion of apoB-48 VLDL.[30] In parallel, HFr decreased phospho-ACC/ACC protein ratio and increased IHTG-DNPalm content, implying on stimulated hepatic DNL. These effects of fructose on hepatic DNL were already observed in human studies.,[31,32] where increased DNL represents a distinct characteristic of individuals with NAFLD.[33] In line with the stimulation of DNL, hepatic FA composition analysis demonstrated increased palmitate, the main product of DNL, as well as augmented C16 and C18 desaturase indexes and increased oleate,[34,35] which is in accordance with the previous notion that fructose promotes de novo synthesis of saturated FAs and oleate.[36] It has been previously demonstrated that hepatic DNL correlates with fDNL in VLDL.[37] This was confirmed in our study, since both fDNL-VLDL and VLDL-DNPalm secretion were increased by dietary fructose.[38] However, the lipogenic effect of fructose is not limited exclusively to provision of excess substrates for DNL, but may also be the consequence of altered FA oxidation and esterification, along with inflammatory processes.[39] Moreover, the susceptibility of adolescent male rats to liver injury induced by fructose intake highly depends on maternal nutrition, since it is demonstrated that consumption of a high-fat diet by pregnant rats led to a reduction of antioxidant defense and impaired activation of the repair mechanisms in the liver of their offspring.[40]

Besides the observation that HFr induces fDNL, our data provide interesting novel hints on the interrelationship between hepatic DNL, VLDL-TG secretion, and intrahepatic fat concentration depending on the feeding/non-feeding circadian cycle. HFr induced diurnal variations in intrahepatic DNPalm content and fDNL-VLDL, which were increased after the feeding phase and returned toward normal after the non-feeding phase. In contrast, plasma VLDL-TGs were not significantly different between the two phases. This suggests that FAs synthesized from fructose during the feeding phase were in part immediately secreted as VLDL-TG while partially routed toward hepatic deposition, to be subsequently released as VLDL-TG during the non-feeding phase. We propose that fructose-induced intrahepatic TG storage acted as a physiological "buffer" to prevent excessive VLDL-TG increase during the feeding phase, by promoting accumulation of FAs. Noticeably, both elevated peripheral FFA and hepatic DNL may contribute to hepatic fat storage.[37] In our study, HFr also led to increased plasma FFA that could be lipotoxic. IHTG accumulation in HFr animals might thus represent an adaptive hepatoprotective mechanism against FFA-induced lipotoxicity.[41] Interestingly, although plasma FFA and expression of lipogenic enzymes were increased after both 10% and 20% liquid fructose diet, increased intrahepatic triglycerides were present only after 20% fructose supplementation.[16,42] This disparity may be a consequence of different total fructose intake. In addition, it may also be explained by the fact that animals were fasted overnight in the 10% liquid fructose study, but not in the present study. It has indeed been demonstrated that fasting suppresses hepatic lipid accumulation in rats exposed to semi-synthetic diet.[43] The observed diurnal pattern in hepatic DNL is in accordance with previous study demonstrating that HFr diet causes circadian alterations in lipogenic gene expression in rat liver, with maximum amplitude at the beginning of light phase.[44] Similar observa-

tions were reported in humans by Hudgins et al., who found that subjects with a "diurnal" pattern are characterized by a low fasting fDNL followed by a late evening peak and return to low levels the next morning.[35]

A novelty of our study is the observation that HFr influenced lipogenesis in the kidney by increasing: 1) the percent of palmitate in the total renal fat; 2) DNPalm content in IRTG; 3) renal FAS gene expression. However, these changes were not accompanied by intrarenal lipid accumulation. This was possibly due to the fact that IRTG are markedly lower than IHTG and hence our TG assay was not sensitive enough to detect changes. In our study, both apoB and MTTP genes were expressed in the kidney, suggesting that the kidney may actively contribute to VLDL-TG secretion.[45] Interestingly, the effects of HFr on FA profiles differed between liver and kidney. These observations strongly support the hypothesis that kidney contributed actively to DNL in response to HFr. However, the renal contribution to total newly synthesized palmitate was likely to be minor in comparison to hepatic production, taking into account that the level of hepatic palmitate content is 1000-fold higher than renal palmitate and that post-hepatic fructose delivery ranges only between 0–20% of ingested fructose.[3] The functional significance and the potential consequences of fructose-induced renal DNL remain to be further assessed.

In our experiments, CS alone increased blood glucose concentrations, but had no significant effect on glucose tolerance[28] or on intrahepatic/intrarenal fat, fDNL, or VLDL-TG secretion. In contrast, the combined CS and HFr enhanced hypertriglyceridemia and fDNL-VLDL, while CS additionally potentiated the effects of HFr on increased IRTG-DNPalm content at the end of the feeding phase. This was associated with a significantly increased lipogenic gene expression in both liver and kidney as a result of interaction between HFr and CS, suggesting that CS enhanced fructose carbon flux toward FA synthesis through stimulation of both intrahepatic and intrarenal trioses production and DNL activity. CS thus boosted the effects of HFr on VLDL–DNPalm secretion and plasma VLDL-TG, but at the same time temporarily decreasing IHTG storage during the feeding phase, which suggests that newly made palmitate was channeled into the circulation rather than being sequestered in the liver. This may be explained by increased glucocorticoids evoked by chronic stress,[24] since these hormones promote both hepatic TG synthesis and their release as VLDL.[46] In our study, stress significantly increased mRNA level of MTTP, which is a key player in the assembly and secretion of hepatic VLDL. However, this effect was not repeated after combined treatment. On the other hand, protein level of perilipin 2, the other crucial player in the lipid droplet formation and VLDL assembly, did not differ between two HFr groups. This finding implies that reduced intrahepatic TG accumulation and stimulated export of VLDL-TG observed in HFr+CS group was not related to this protein. Therefore, the underlying mechanism is yet to be clarified. An increase in VLDL-TG is generally associated with an increase in LDL-cholesterol and a decrease in HDL-cholesterol, and may represent an independent risk factor for coronary heart diseases and atherosclerosis.[47] Consequently, the interaction of HFr and CS may be particularly relevant for human health since their

**ADVANCED
SCIENCE NEWS**

www.advancedsciencenews.com

**Molecular Nutrition
Food Research**

www.mnf-journal.com

combination may increase cardiometabolic risk. On the other hand, although plasma glucose was increased in HFr+CS animals, CS actually ameliorated the HFr-induced glucose intolerance and restored it to normal level.[48]

This study has several strengths: First, it comprehensively assessed the effects of HFr and CS on DNL by simultaneously measuring fDNL in rats by an isotopic method and through changes in tissue FAs profiles; second, this is, to our knowledge, the first study documenting concurrent incorporation of labeled de novo synthesized palmitate in VLDL-TG, intrahepatic TG, and intrarenal TG; and third, it integrated measurements obtained at the end of the feeding and non-feeding phases, allowing to assess the temporal relationship between fructose intake and its metabolic effects over a 24-hour period. However, it also has some limitations. First, inter-organ lipid exchanges could not be measured; therefore, we cannot discard the premise that storage of de novo synthesized lipids in the kidney was secondary to kidney uptake of VLDL-TGs synthesized in the liver. Our observation that FA profile differed in the liver and kidney, however, supports the hypothesis that intrarenal lipids were at least in part locally synthesized. Second, we obtained measurements at the end of the feeding and non-feeding phases, but did not actually assess how these evolved over time, for example, we did not measure how long VLDL-TG and newly synthesized palmitate secretion remain elevated after the end of the feeding phase. Third, the use of 20% fructose solution resulted in daily fructose intake that is unlikely to be observed in regular human diets. However, it was deliberately chosen as it allows the measurement of substrate fluxes with isotopic methods. Finally, the fact that fructose in sweetened beverages is usually co-ingested with glucose should not be neglected, as well. It was previously shown that glucose may aggravate the fructose-mediated effects by facilitating absorption of fructose,[49] significantly decreasing fructose oxidation and gluconeogenesis, and increasing fructose carbon storage.[50]

In summary, our data indicate that an HFr increases glucose and VLDL-TG (the latter being a result of stimulated DNL in both liver and kidney), increases fDNL-VLDL, and decreases VLDL-TG clearance. In addition, liquid high-fructose supplementation is associated with a temporary storage of de novo synthesized fat in the liver during the feeding phase, followed by a mobilization of intrahepatic fat in the non-feeding phase. This temporary fat storage may serve as a physiological "buffer" that prevents excess release of VLDL-TG in the systemic circulation during the feeding phase. Chronic stress alone increases glucose, but does not alter lipid metabolism. In contrast, chronic stress associated with high-fructose supplementation markedly enhances VLDL-TG concentration. The proposed mechanisms rely on interaction of chronic stress and fructose on fDNL and a stimulation of newly synthesized palmitate secretion in the form of VLDL, resulting in a decreased temporary storage of intrahepatic fat and enhanced secretion of VLDL-TG. In addition, our results indicate that HFr also induces alterations of both glucose and lipid metabolism in the kidney. However, the need for further assessment of the role of the kidney in fructose-induced metabolic alterations remains. In conclusion, our results showed that the combination of HFr and chronic stress, as hallmarks of modern lifestyle, exerts more detrimental influence on lipid homeostasis than the individual factors, judged by stimulated fDNL and increased export of VLDL-TGs to non-hepatic tissues.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

L.T., G.M., N.V., and A.Dj. designed the research. A.T., B.B., M.N., S.K., Lj.G., N.V., D.V.M., J.B., and A.Dj. performed experiments. N.V. and D.V.M. analyzed the data. N.V. wrote the manuscript. F.P. contributed to the discussion. N.V. is responsible for the integrity of the work as a whole. All authors reviewed and approved the final manuscript.

[1] M. J. Dekker, Q. Su, C. Baker, A. C. Rutledge, K. Adeli, *Am. J. Physiol. Endocrinol. Metab.* **2010**, *299*, E685.

[2] M. A. Herman, V. T. Samuel, *Trends Endocrinol. Metab.: TEM* **2016**, *27*, 719.

[3] C. Francey, J. Cros, R. Rosset, C. Creze, V. Rey, N. Stefanoni, P. Schneiter, L. Tappy, K. Seyssel, *Clin. Nutr. ESPEN* **2019**, *29*, 125.

[4] A. Gonzalez-Vicente, J. L. Garvin, U. Hopfer, *PLoS One* **2018**, *13*, e0201293.

[5] O. Björkman, R. Gunnarsson, E. Hagström, P. Felig, J. Wahren, *J. Clin. Invest.* **1989**, *83*, 52.

[6] G. Musso, M. Cassader, S. Cohney, S. Pinach, F. Saba, R. Gambino, *Trends Mol. Med.* **2015**, *21*, 645.

[7] J. D. Horton, J. L. Goldstein, M. S. Brown, *J. Clin. Invest.* **2002**, *109*, 1125.

[8] P. W. Caton, N. K. Nayuni, N. Q. Khan, E. G. Wood, R. Corder, *J. Endocrinol.* **2011**, *208*, 273.

[9] K. A. Scott, S. J. Melhorn, R. R. Sakai, *Curr. Obes. Rep.* **2012**, *1*, 16.

[10] G. P. Chrousos, *Int. J. Obes. Relat. Metab. Disord.* **2000**, *24*, S50.

[11] U. Lemke, A. Krones-Herzig, M. Berriel Diaz, P. Narvekar, A. Ziegler, A. Vegiopoulos, A. C. Cato, S. Bohl, U. Klingmuller, R. A. Screaton, K. Muller-Decker, S. Kersten, S. Herzig, *Cell Metab.* **2008**, *8*, 212.

[12] Y. Krausz, H. Bar-On, E. Shafrir, *Biochim. Biophys. Acta* **1981**, *663*, 69.

[13] A. Teofilović, J. Brkljačić, A. Djordjevic, D. Vojnović Milutinović, L. Tappy, G. Matić, N. Veličković, *Int. J. Food Sci. Nutr.* **2020**, 1.

[14] P. G. Reeves, F. H. Nielsen, G. C. Fahey, Jr., *J. Nutr.* **1993**, *123*, 1939.

[15] M. Joels, H. Karst, D. Alfarez, V. M. Heine, Y. Qin, E. van Riel, M. Verkuyl, P. J. Lucassen, H. J. Krugers, *Stress* **2004**, *7*, 221.

[16] A. Vasiljević, N. Veličković, B. Bursać, A. Djordjevic, D. V. Milutinović, N. Nestorović, G. Matić, *J. Nutr. Biochem.* **2013**, *24*, 1790.

[17] S. M. Turner, E. J. Murphy, R. A. Neese, F. Antelo, T. Thomas, A. Agarwal, C. Go, M. K. Hellerstein, *Am. J. Physiol.: Endocrinol. Metab.* **2003**, *285*, E790.

[18] E. J. Murphy, *J. Anim. Sci.* **2006**, *84*, E94.

[19] G. Asher, P. Sassone-Corsi, *Cell* **2015**, *161*, 84.

[20] V. Matyash, G. Liebisch, T. V. Kurzchalia, A. Shevchenko, D. Schwudke, *J. Lipid Res.* **2008**, *49*, 1137.

[21] M. Rasouli, H. Tahmouri, M. Mosavi-Mehr, *J. Clin. Diagn. Res.* **2016**, *10*, BF01.

[22] B. J. McCabe, I. R. Bederman, C. Croniger, C. Millward, C. Norment, S. F. Previs, *Anal. Biochem.* **2006**, *350*, 171.

[23] K. Minehira, S. G. Young, C. J. Villanueva, L. Yetukuri, M. Oresic, M. K. Hellerstein, R. V. Farese, Jr., J. D. Horton, F. Preitner, B. Thorens, L. Tappy, *J. Lipid Res.* **2008**, *49*, 2038.

[24] N. Veličković, A. Teofilović, D. Ilić, A. Djordjevic, D. Vojnović Milutinović, S. Petrović, F. Preitner, L. Tappy, G. Matić, *Eur. J. Nutr.* **2018**, *58*, 1829.

[25] N. Veličković, A. Djordjevic, A. Vasiljević, B. Bursać, D. V. Milutinović, G. Matić, *Br. J. Nutr.* **2013**, *110*, 456.

[26] B. Bjorndal, L. Burri, V. Staalesen, J. Skorve, R. K. Berge, *J. Obes.* **2011**, *2011*, 490650.

[27] M. Dirlewanger, P. Schneiter, E. Jequier, L. Tappy, *Am. J. Physiol.: Endocrinol. Metab.* **2000**, *279*, E907.

[28] N. Veličković, A. Teofilović, D. Ilić, A. Djordjevic, D. Vojnović Milutinović, S. Petrović, F. Preitner, L. Tappy, G. Matić, *Eur. J. Nutr.* **2019**, *58*, 1829.

[29] T. L. Jensen, M. K. Kiersgaard, D. B. Sorensen, L. F. Mikkelsen, *Lab. Anim.* **2013**, *47*, 225.

[30] B. Magnusson, L. Asp, P. Bostrom, M. Ruiz, P. Stillemark-Billton, D. Linden, J. Boren, S. O. Olofsson, *Arterioscler., Thromb., Vasc. Biol.* **2006**, *26*, 1566.

[31] D. Faeh, K. Minehira, J. M. Schwarz, R. Periasamy, S. Park, L. Tappy, *Diabetes* **2005**, *54*, 1907.

[32] J. M. Schwarz, S. M. Noworolski, M. J. Wen, A. Dyachenko, J. L. Prior, M. E. Weinberg, L. A. Herraiz, V. W. Tai, N. Bergeron, T. P. Bersot, M. N. Rao, M. Schambelan, K. Mulligan, *J. Clin. Endocrinol. Metab.* **2015**, *100*, 2434.

[33] J. E. Lambert, M. A. Ramos-Roman, J. D. Browning, E. J. Parks, *Gastroenterology* **2014**, *146*, 726.

[34] M. Baena, G. Sanguesa, A. Davalos, M. J. Latasa, A. Sala-Vila, R. M. Sanchez, N. Roglans, J. C. Laguna, M. Alegret, *Sci. Rep.* **2016**, *6*, 26149.

[35] L. C. Hudgins, M. K. Hellerstein, C. E. Seidman, R. A. Neese, J. D. Tremaroli, J. Hirsch, *J. Lipid Res.* **2000**, *41*, 595.

[36] J. C. P. Silva, C. Marques, F. O. Martins, I. Viegas, L. Tavares, M. P. Macedo, J. G. Jones, *Metab. Eng.* **2019**, *56*, 69.

[37] K. L. Donnelly, C. I. Smith, S. J. Schwarzenberg, J. Jessurun, M. D. Boldt, E. J. Parks, *J. Clin. Invest.* **2005**, *115*, 1343.

[38] L. C. Hudgins, T. S. Parker, D. M. Levine, M. K. Hellerstein, *J. Clin. Endocrinol. Metab.* **2011**, *96*, 861.

[39] J. B. Moore, P. J. Gunn, B. A. Fielding, *Nutrients* **2014**, *6*, 5679.

[40] L. S. Oliveira, B. Caetano, R. A. Miranda, A. F. P. Souza, A. Cordeiro, J. Woyames, C. B. V. Andrade, G. C. Atella, C. M. Takiya, R. S. Fortunato, I. H. Trevenzoli, L. L. Souza, C. C. Pazos-Moura, *Mol. Nutr. Food Res.* **2020**, *64*, e1900838.

[41] N. Alkhouri, L. J. Dixon, A. E. Feldstein, *Expert Rev. Gastroenterol. Hepatol.* **2009**, *3*, 445.

[42] J. Brkljačić, N. Veličković, I. Elaković, A. Teofilović, D. Vojnović Milutinović, A. Djordjevic, G. Matić, *Arch. Biol. Sci.* **2019**, *71*, 417.

[43] I. Ikeda, K. Metoki, T. Yamahira, M. Kato, N. Inoue, K. Nagao, T. Yanagita, H. Shirakawa, M. Komai, *Biosci., Biotechnol., Biochem.* **2014**, *78*, 1584.

[44] S. Sun, F. Hanzawa, D. Kim, M. Umeki, S. Nakajima, K. Sakai, S. Ikeda, S. Mochizuki, H. Oda, *J. Biol. Chem.* **2019**, *294*, 15206.

[45] M. Krzystanek, T. X. Pedersen, E. D. Bartels, J. Kjaehr, E. M. Straarup, L. B. Nielsen, *J. Biol. Chem.* **2010**, *285*, 10583.

[46] D. P. Macfarlane, S. Forbes, B. R. Walker, *J. Endocrinol.* **2008**, *197*, 189.

[47] S. M. Grundy, *Am. J. Cardiol.* **1998**, *81*, 18B.

[48] G. Balsevich, A. Uribe, K. V. Wagner, J. Hartmann, S. Santarelli, C. Labermaier, M. V. Schmidt, *J. Endocrinol.* **2014**, *222*, 15.

[49] K. L. Stanhope, S. C. Griffen, B. R. Bair, M. M. Swarbrick, N. L. Keim, P. J. Havel, *Am. J. Clin. Nutr.* **2008**, *87*, 1194.

[50] F. Theytaz, S. de Giorgi, L. Hodson, N. Stefanoni, V. Rey, P. Schneiter, V. Giusti, L. Tappy, *Nutrients* **2014**, *6*, 2632.