JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2021, 72, 1, 45-53
www.jpp.krakow.pl | DOI: 10.26402/jpp.2021.1.05

J. KOVAR[1], T. DUSILOVA[1,2], P. SEDIVY[1], R. BRUHA[3], H. GOTTFRIEDOVA[1], P. PAVLIKOVA[1], J. PITHA[1], V. SMID[3], M. DROBNY[1], M. DEZORTOVA[1], M. HAJEK[1]

ACUTE RESPONSES OF HEPATIC FAT CONTENT TO CONSUMING FAT, GLUCOSE AND FRUCTOSE ALONE AND IN COMBINATION IN NON-OBESE NON-DIABETIC INDIVIDUALS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

[1]Institute for Clinical and Experimental Medicine, Prague, Czech Republic; [2]Department of Physiology, Faculty of Science, Charles University, Prague, Czech Republic; [3]Fourth Department of Internal Medicine, First Faculty of Medicine and General University Hospital, Charles University, Prague, Czech Republic

We have recently demonstrated that a high-fat load can induce immediate increase in hepatic fat content (HFC) and that such an effect can be modified differently by co-administration of fructose or glucose in healthy subjects. Therefore, we addressed the question how consumption of these nutrients affects changes in HFC in subjects with non-alcoholic fatty liver disease (NAFLD). Eight male non-obese non-diabetic patients with NAFLD underwent 6 experiments each lasting 8 hours: 1. fasting, 2. high-fat load (150 g of fat (dairy cream) at time 0), 3. glucose (three doses of 50 g at 0, 2, and 4 hours), 4. high-fat load with three doses of 50 g of glucose, 5. fructose (three doses of 50 g at 0, 2, and 4 hours), 6. high-fat load with three doses of 50 g of fructose. HFC was measured using magnetic resonance spectroscopy prior to meal administration and 3 and 6 hours later. Plasma triglycerides, non-esterified fatty acids, glucose and insulin were monitored throughout each experiment. HFC increased by 10.4 ± 6.9% six hours after a high-fat load and by 15.2 ± 12.5% after high-fat load with fructose. When co-administering glucose with fat, HFC rose only transiently to return to baseline at 6 hours. Importantly, NAFLD subjects accumulated almost five times more fat in their livers than healthy subjects with normal HFC. Consumption of a high-fat load results in fat accumulation in the liver of NAFLD patients. Fat accumulation after a fat load is diminished by glucose but not fructose co-administration.

Key words: *non-alcoholic fatty liver disease, hepatic fat content, glucose, fructose, dietary fat, insulin, proton magnetic resonance spectroscopy*

INTRODUCTION

The epidemic of non-alcoholic fatty liver disease (NAFLD) has recently become a major problem facing health systems worldwide (1-3). Hepatic triglycerides (TG) come from three principal sources: non-esterified fatty acids (NEFA) released into the circulation from adipose tissue, dietary fat (entering the liver both in chylomicron remnants and as spillover NEFA) and *de novo* lipogenesis (DNL) from simple carbohydrates and amino acids. In the liver, some fatty acids enter the mitochondria and serve as a substrate for β-oxidation or synthesis of ketone bodies and the bulk of fatty acids is relatively quickly secreted from the liver as very low density lipoprotein-TG (VLDL-TG) to be stored or oxidized in extrahepatic tissues (4). Therefore, fat does not accumulate in a healthy liver.

The pathways responsible for fat accumulation in the liver in patients with NAFLD have been thoroughly studied and it was demonstrated that DNL is a critical factor contributing to hepatic fat accumulation (5, 6). However, in these studies based on sophisticated techniques using stable isotopes, the role of different sources in hepatic fat accumulation was established through analysis of TG secreted from the liver and analysis of fat composition in liver biopsy samples; these studies did not quantify the changes in hepatic fat content (HFC). These changes can be measured directly using methods of magnetic resonance (7). Using proton magnetic resonance spectroscopy (^{1}H-MRS), we recently demonstrated in healthy subjects *in vivo* that changes in HFC can be observed within a few hours after administration of a sufficiently large load of dietary fat or sugars (8). We found that HFC increases after a fat load and that the accumulation of fat can be prevented by glucose, but not fructose co-administration. It remains to be clarified whether the same applies to patients with steatosis. Therefore, in this study, we analyzed the acute response of liver fat to different nutrients in subjects with NAFLD. To eliminate the role of manifest metabolic syndrome that can be involved in steatosis development, the study was conducted in non-diabetic subjects who were not obese and did not differ from previously studied healthy subjects (8) in age, BMI, and fasting plasma glucose. Six experiments lasting 8 hours each that included different dietary interventions (fasting, high-fat load, repeated loads of glucose or fructose with or without a high-fat load) were carried out in all

study participants. Hepatic fat content was quantified three times during each experiment using ^{1}H-MRS.

SUBJECTS AND METHODS

Subjects

The study was carried out in eight non-obese, non-diabetic, and otherwise healthy male subjects with liver steatosis (HFC higher than 5%). The subjects with NAFLD confirmed by ^{1}H-MRS who were treated only by lifestyle intervention and subjects with steatosis accidentaly detected during abdominal ultrasound were referred by cooperating physicians. The exclusion criteria were BMI above 30 kg/m^2, fasting plasma glucose above 5.9 mmol/L, diagnosis of diabetes mellitus, glucose concentration above 11 mmol/L at both 1 and 2 hours of oral glucose tolerance test, use of pharmacological agents affecting insulin sensitivity and lipid metabolism, reported alcohol consumption above 140 g/wk, and inability to undergo the ^{1}H-MRS examination. The subjects were age- and BMI-matched to those with normal HFC from our previous study (8). A total 49 subjects with suspected NAFLD were contacted, 22 declined to participate and 19 did not meet the inclusion criteria for the study. Participants were recruited from August 2016 to November 2019. The diagnosis of NAFLD was based on ^{1}H-MRS-documented hepatic steatosis (HFC higher than 5% at the first examination) and the absence of secondary causes of hepatic fat accumulation (9). Prior to inclusion in the study, seven out of the eight included patients underwent liver shear wave elastography showing no signs of fibrosis.

Experimental design

The design of the study was identical to that of our study in healthy subjects without steatosis (8). Each of the 8 subjects underwent six experiments each lasting 8 hours that differed in the diet consumed during the experiment. All experiments started in the morning after an overnight fast by ^{1}H-MRS examination for HFC determination. Next, a cannula for blood draws was inserted into the antecubital vein and the first blood sample taken; the subjects subsequently consumed experimental breakfast (time 0). Hepatic fat content was determined by ^{1}H-MRS again at 3 and 6 hours after the experimental breakfast. Blood samples for TG, NEFA, glucose, and insulin determination were subsequently drawn at 0.5, 1, 2, 2.5, 3, 4, 4.5, 5 and 6 hours in each experiment. The six dietary interventions were as follows:

- fasting (Fasting experiment);
- a high-fat load alone (460 ml of dairy cream containing 150 g of fat, 19.5 g of carbohydrate, and 13.5 g of protein,1460 kcal total) administered at time 0 h (Fat experiment);
- 50 g of glucose dissolved in fruit tea administered at 0, 2, and 4 hours (600 kcal total) (Glucose experiment);
- a high-fat load at time 0 + 50 g of glucose at 0, 2, and 4 hours (Fat + Glucose experiment);
- 50 g of fructose dissolved in fruit tea administered at 0, 2, and 4 hours (600 kcal total) (Fructose experiment);
- a high-fat load at time 0 + 50 g of fructose at 0, 2, and 4 hours (Fat + Fructose experiment) (*Fig. 1*).

The order of the experiments was randomized and the interval between individual experiments was at least 6 days (median interval 15 days). The subjects were asked not to change their lifestyle and eating habits throughout the study. In each of the experiments, information on the physical activity and food on the day before the experiment and any changes between the experiments was obtained. In each experiment, weight, waist and hip circumferences were also measured and the body fat content and body composition were determined by bioelectrical impedance analysis (BODYSTAT® 1500, Bodystat Ltd., Douglas, Isle of Man). Moreover, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), and C-reactive protein (CRP) were determined in baseline fasting samples (time 0) to check the health status of each subject.

Magnetic resonance measurements

Hepatic fat content was measured by *in vivo* ^{1}H-MRS. Examinations were performed on a 3T whole-body scanner (3T Trio, Siemens, Erlangen, Germany) equipped with an eight-channel surface body coil. Automatic and manual shimming were combined to reach a linewidth below 50 Hz. Standard PRESS (Point REsolved Spectroscopy Sequence) single-voxel spectroscopy was used (echo time (TE)) 30 ms and repetition time (TR) 4500 ms) to measure HFC. Voxel size of our volume of interest (VOI) was set at 40 × 30 × 25 mm and the same VOI position in liver segments V/VIII was carefully checked during all subsequent examinations. One spectrum acquisition during each breath-hold was obtained with the measurement repeated three times. Relaxation times were measured using the same sequence (PRESS) with echoes of 30, 50, 68, 135, 180 and 270 ms. In one subject, HFC measurement was performed on a 3T whole-body VIDA scanner (Siemens, Erlangen, Germany) using the same protocol.

Calculations of T2 relaxation times were done using MATLAB software (Mathworks, Natick, MA, USA). T2 values of water and lipid CH2 groups were found in the range 30 ± 3 ms and 53 ± 11 ms without any extreme values and these T2s are in agreement with literature data. T1 values were not calculated as only one acquisition spectrum was measured.

Spectra were evaluated using LCModel version 6.2 (http://s-provencher.com/lcmodel.shtml). Lipid signals of aliphatic protons in the range of 0.0 – 3.0 ppm (–CH_3 0.8 – 0.9 ppm;

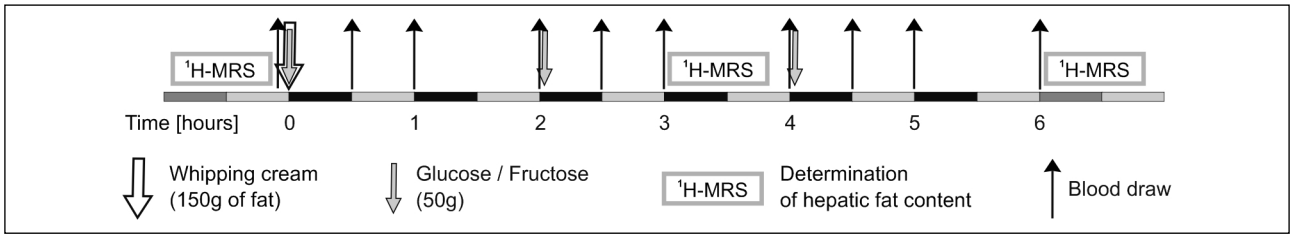

Fig. 1. Study design. Eight male patients with liver steatosis underwent 6 experiments each. Hepatic fat content (HFC) was determined three times during each experiment using ^{1}H-MRS and 10 blood samples were taken. The experiments differed in the type of food administered. In each experiment, subjects received one of the following interventions at the times indicated above:
(1) nothing (fasting); (2) high-fat load (150 g of fat (cream)); (3) 3 × 50 g of glucose; (4) high-fat load and 3 × 50 g of glucose; (5) 3 × 50 g of fructose; (6) high-fat load and 3 × 50 g of fructose.

$–(CH_2)_n$ 1.2 – 1.4 ppm; $–CH_2CH_2CH=CH$ 1.9 – 2.1 ppm; $–CH=CH –CH_2 –CH=CH$ 2.8 ppm) and water and olefinic proton signals (4.7 and 5.3 ppm) were fitted as described in an earlier paper (10). The percentage of HFC was calculated according to Longo (11).

Biochemical analysis

Blood was collected into vacutainers with EDTA and immediately chilled on ice. The aliquots of plasma were subsequently stored at –80ºC until analysis. Triglyceride concentrations were determined using enzymatic kits manufactured by Roche Diagnostics (Mannheim, Germany), glucose concentrations using a PLIVA Lachema Diagnostika kit (Brno, Czech Republic) and NEFA were measured using a kit manufactured by Wako Chemicals GmbH (Neuss, Germany). Insulin was measured using an IRMA kit (Beckman Coulter, Prague, Czech Republic).

DNA analysis

DNA was isolated from peripheral blood using a Qiagen QIAamp kit (Qiagen, Hilden, Germany). The rs58542926 locus in *TM6SF2* was analyzed using TaqMan SNP assay No. C_89463510_10 (Thermo Fisher Scientific, Waltham, MA, USA). The rs738409 locus in *PNPLA3* was genotyped with TaqMan SNP assay No. C_7241_10. Genotyping was performed according to the manufacturer's protocol. Both assays were run on the Applied Biosystems ABI 7300 Real-Time PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA).

Statistics

Data are presented as mean ± SD. The areas under the increment curve (AUICs) for biochemical parameters in the plasma were calculated using the trapezoid rule after adjustment for baseline values. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Hepatic fat content expressed as percentage of baseline value was compared at three time points (0, 3, and 6 hours) during each experiment using ANOVA for repeated measures and Dunnett's multiple comparison post-test. The HFC responses to test meals were also compared with those of previously studied healthy subjects (8) using mixed model ANOVA (mm ANOVA) for repeated measures with one grouping factor - steatosis, using JMP 11.0 software (SAS Institute Inc., Cary, NC, USA).

Changes in biochemical parameters over time (TG, NEFA, glucose and insulin) were evaluated using ANOVA for repeated measures and Dunnett's multiple comparison post-test. AUIC values of biochemical parameters were compared using repeated measures ANOVA and Tukey's multiple comparison post-test. The one-sample t-test was used to determine the AUIC difference from zero. A $p < 0.05$ was considered statistically significant.

Table 1. Characteristics of subjects included in the study.

Number	**8**
Age at study entry (years)	40.0 ± 7.6
BMI (kg/m^2)	27.2 ± 2.2
Waist circumference (cm)	95.9 ± 7.8
Waist/hip ratio	0.94 ± 0.05
Total body fat (kg)	21.7 ± 2.8
Hepatic fat content (%)	12.8 ± 7.3
Triglycerides (mmol/L)	1.7 ± 0.7
Cholesterol (mmol/L)	4.8 ± 0.9
Non-esterified fatty acids (NEFA) after overnight fast (mmol/L)	0.30 ± 0.07
Fasting glucose (mmol/L)	5.6 ± 0.4
Fasting insulin (μU/mL)	10.0 ± 4.5
HOMA-IR	2.5 ± 1.1
HbA1c (mmol/mol)	36.5 ± 4.4
Gamma-glutamyltransferase (GGT) (μkat/L)	0.83 ± 0.44
Aspartate aminotransferase (AST) (μkat/L)	0.45 ± 0.22
Alanine aminotransferase (ALT) (μkat/L)	0.80 ± 0.22

Data are mean ± SD.

Study approval

All participants gave written informed consent prior to inclusion in the study, which had been approved by the Joint Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital in Prague, Czech Republic. All work was conducted in compliance with the principles of the Declaration of Helsinki.

The study was registered at ClinicalTrials.gov (NCT03680248).

RESULTS

Baseline characteristics of subjects

The subjects included in the study were overweight but non-obese (BMI ≤ 30 kg/m^2) and had normal plasma lipid, glucose and insulin concentrations (*Table 1*). Impaired glucose tolerance during oral glucose tolerance test was detected in 2 subjects and one of them had also slightly elevated HbA_{1c} (44 mmol/mol). Increased GGT activity (above 1.07 μkat/L) was found in 5 subjects, increased activities of AST (above 0.75 μkat/L) and ALT (above 1.17 μkat/L) were found in 1 and 3 subjects, respectively. One subject was homozygous and two subjects heterozygous carriers of the minor allele rs738409, C→G of the *PNPLA3* gene; one subject carried the minor allele rs58542926, C→T of the *TM6SF2* gene; one subject carried both minor alleles; and three subjects were homozygous carriers of major variants of both genes.

Each of the eight subjects underwent all six experiments. The baseline values of parameters reflecting the metabolic status and liver function of subjects (TG, NEFA, glucose, insulin, GGT, AST, and ALT) measured at the beginning of each of the six experiments did not differ between the experiments.

The subjects in the study did not differ in age, BMI, waist circumference, waist/hip ratio, percentage of total body fat,

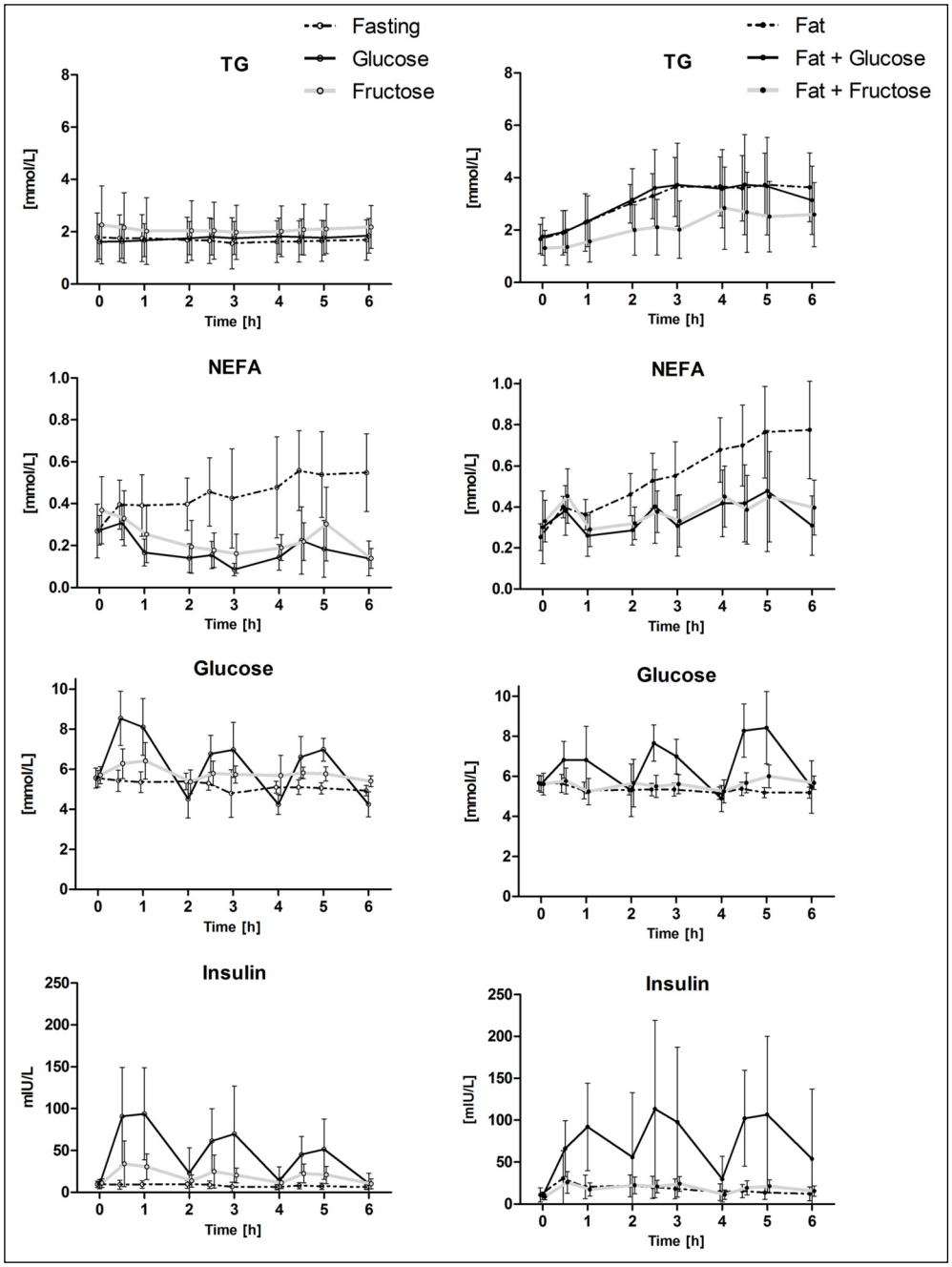

Fig. 2. Dynamics of plasma TG, NEFA, glucose, and insulin concentrations during the six experiments: Fasting; Fat (150 g of fat in dairy cream at 0 hour); Glucose (3 × 50 g of glucose at 0, 2 and 4 hours); Fat + Glucose (150 g of fat at 0 hour and 3 × 50 g of glucose at 0, 2 and 4 hours); Fructose (3 × 50 g of fructose at 0, 2 and 4 hours); Fat + Fructose (150 g of fat at 0 hour and 3 × 50 g of fructose at 0, 2 and 4 hours). Experiments without a fat load are presented in the left panels, experiments with a fat load in the right panels. Nutrients were ingested after blood draws at indicated times. All experiments were carried out in 8 patients with NAFLD. Data are mean ± SD. NEFA, non-esterified fatty acids; TG, triglycerides.

cholesterol, fasting glucose and NEFA concentrations from healthy volunteers with a normal liver fat content in our recent study (8). They had only slightly but significantly higher fasting TG and insulin concentrations and HOMA-IR.

Fasting has no effect on hepatic fat content

In a control experiment with no food administered to subjects (Fasting experiment), plasma TG did not change, glucose and insulin slightly dropped, whereas NEFA concentrations rose steadily to double baseline values (from 0.27 ± 0.13 to 0.55 ± 0.19 mmol/L ($p < 0.001$) at the end of the experiment (*Fig. 2*). The changes in these parameters also get reflected in their corresponding AUICs (*Fig. 3*). Hepatic fat content was not affected by prolonged fasting (*Fig. 4*).

A high-fat load induces an increase in hepatic fat content

Administration of 150 g of fat (Fat experiment) increased triglyceridemia rising for 3 hours to remain increased until the end of the experiment (*Fig. 2*). Glucose decreased marginally during the experiment whereas insulin concentrations modestly rose half an hour after fat consumption and then slowly returned to baseline. Non-esterified fatty acid concentrations increased steadily up to three times the baseline at the end of the experiment (*Fig. 2*). Hepatic fat content rose by 10.4% ($p = 0.006$), from 11.9 ± 7.8% to 13.1 ± 8.6%.

Repeated loads of glucose do not affect hepatic fat content

In subjects receiving three 50 g doses of glucose at 0, 2 and 4 hours (Glucose experiment), corresponding peaks in plasma glucose and insulin concentrations were observed at 0.5, 2.5, and 4.5 hours. Glucose administration resulted in a slight increase in triglyceridemia and marked suppression of NEFA concentrations as documented by the corresponding changes in AUICs (TG AUIC +0.87 ± 0.86 mmol/L/6 h, NEFA AUIC –0.61 ± 0.49 mmol/L/6 h) (*Fig. 3*). Importantly, glucose consumption had no impact on HFC.

Repeated loads of fructose also do not affect hepatic fat content

Administration of three 50 g doses of fructose (Fructose experiment) induced insulin response but the insulin AUIC

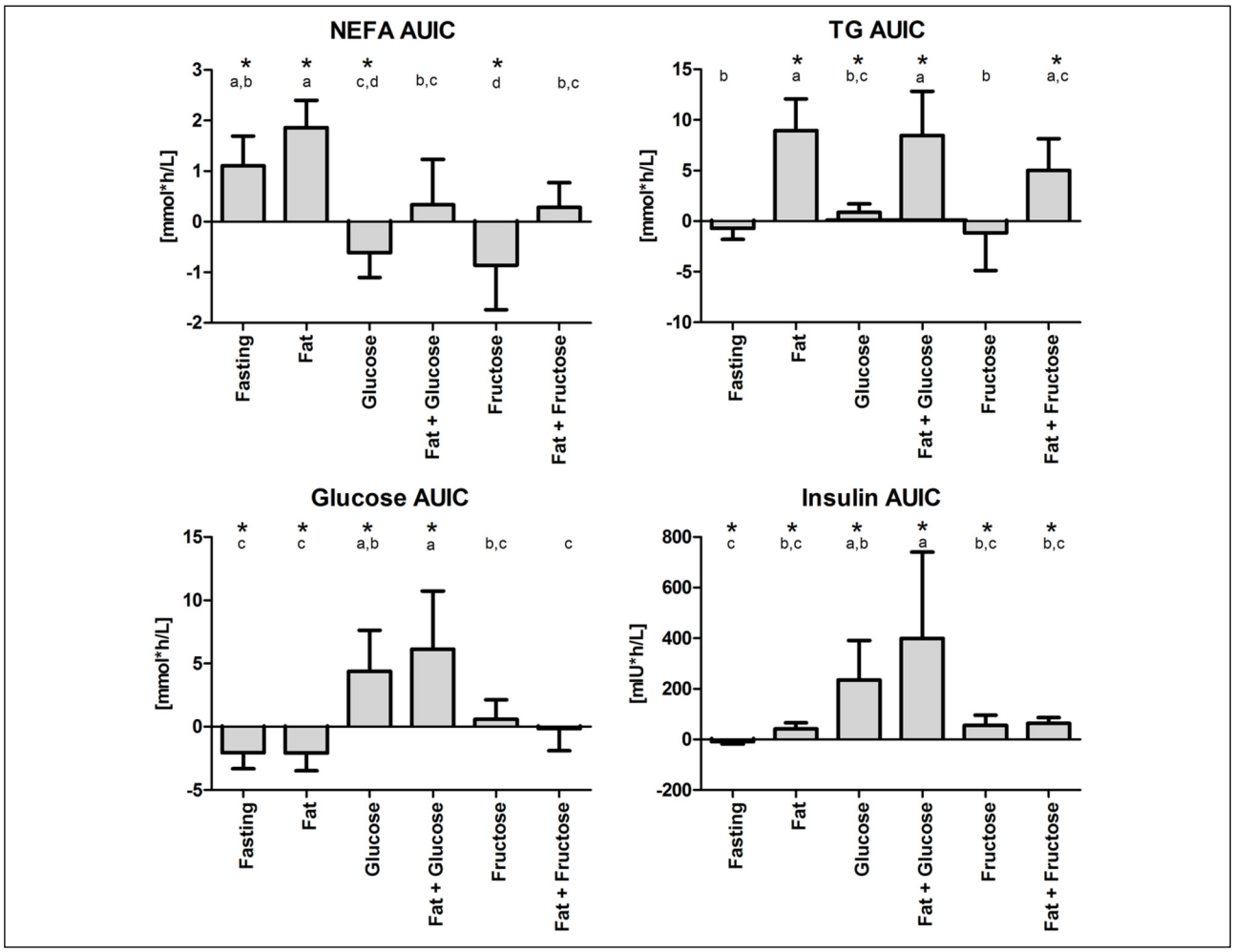

Fig. 3. Areas under the 6-hour increment curves (AUICs) of TG, NEFA, glucose, and insulin during the six experiments. Fasting; Fat (150 g of fat in dairy cream at 0 hour); Glucose (3 × 50 g of glucose at 0, 2 and 4 hours); Fat + Glucose (150 g of fat at 0 hour and 3 × 50 g of glucose at 0, 2 and 4 hours); Fructose (3 × 50 g of fructose at 0, 2 and 4 hours); Fat + Fructose (150 g of fat at 0 hour and 3 × 50 g of fructose at 0, 2 and 4 hours). All experiments were carried out in 8 patients with NAFLD. Data are mean ± SD. * $p < 0.05$ statistically significantly different from zero by the one-sample t-test. The same letter indicates values that do not differ using repeated measures ANOVA and Tukey's multiple comparison post-test. NEFA, non-esterified fatty acids; TG, triglycerides.

was lower by three quarters than that after glucose. Plasma glucose marginally increased, TG concentrations did not change and NEFA were suppressed during the experiment. The response of these three parameters did not differ from that in Glucose experiment when evaluated as AUICs (*Fig. 3*). Importantly, HFC was not affected by repeated loads of fructose.

Co-administration of glucose with a high-fat load transiently increases hepatic fat content

Co-administration of three 50 g doses of glucose with fat (Fat + Glucose experiment) induced corresponding peaks in plasma glucose and insulin concentrations; both glucose and insulin AUICs did not differ from those after glucose alone (*Fig. 3*). Plasma TG concentrations increased and TG AUIC did not differ from that after fat alone. Non-esterified fatty acid concentrations did not change during the experiment. Hepatic fat content rose transiently from 13.8 ± 8.2% to 14.8 ± 7.8% at 3 hours to subsequently return to baseline (14.1 ± 7.8%) at the end of the experiment (*Fig. 4*).

Co-administration of fructose with a high-fat load increases hepatic fat content

Fructose co-administration with fat (Fat + Fructose experiment) did not affect plasma glucose concentrations and induced only a small increase in insulin concentration as documented by AUICs (*Fig. 3*). Triglyceridemia increased similarly as in the Fat and Fat + Glucose experiments. The non-esterified fatty acid concentrations did not change during the experiment as did not in Fat + Glucose experiment. Importantly, HFC rose by 15.2 ± 12.5% (p = 0.014) from 11.5 ± 5.9% to 12.8 ± 5.5% at 6 hours.

Intra-individual biological variability of hepatic fat content

As we measured HFC within a relatively short time span in subjects not intervened in any way, our data could be used to estimate the intra-individual biological variability of HFC. For the calculations, we used data from the morning baseline HFC measurements carried out within 6 months in each of the subjects (5 to 6 measurements per subject) showing an intra-individual

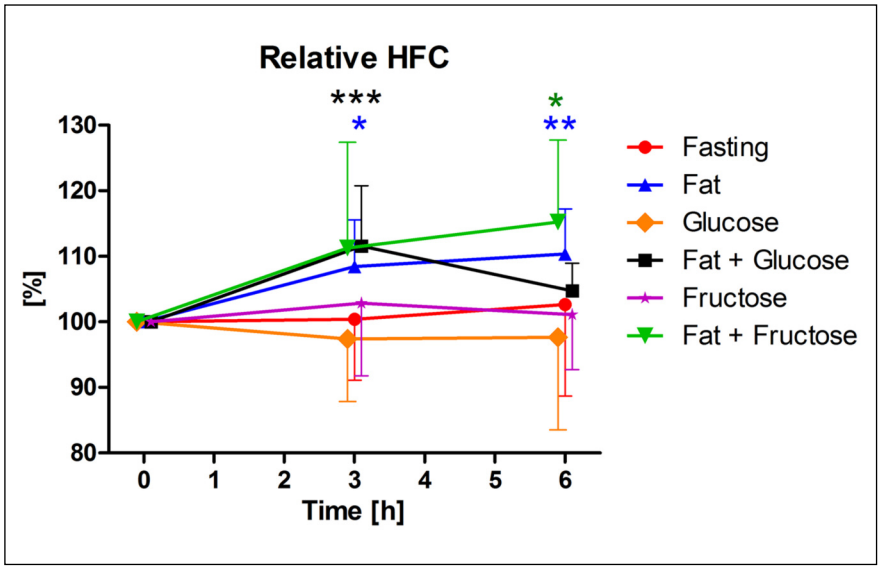

Fig. 4. Dynamics of hepatic fat content (HFC) during the six experiments. Fasting; Fat (150 g of fat in dairy cream at 0 hour); Glucose (3 × 50 g of glucose at 0, 2 and 4 hours); Fat + Glucose (150 g of fat at 0 hour and 3 × 50 g of glucose at 0, 2 and 4 hours); Fructose (3 × 50 g of fructose at 0, 2 and 4 hours); Fat + Fructose (150 g of fat at 0 hour and 3 × 50 g of fructose at 0, 2 and 4 hours). ^{1}H-MRS for HFC determination was carried out before ingestion of cream (time 0) and 3 and 6 hours later. All experiments were carried out in 8 patients with NAFLD. Data expressed as a percentage of baseline morning values are mean ± SD. *p < 0.05, ** p < 0.01, ***p<0.001 significantly different from baseline (ANOVA for repeated measures and Dunnett's multiple comparison post-test).

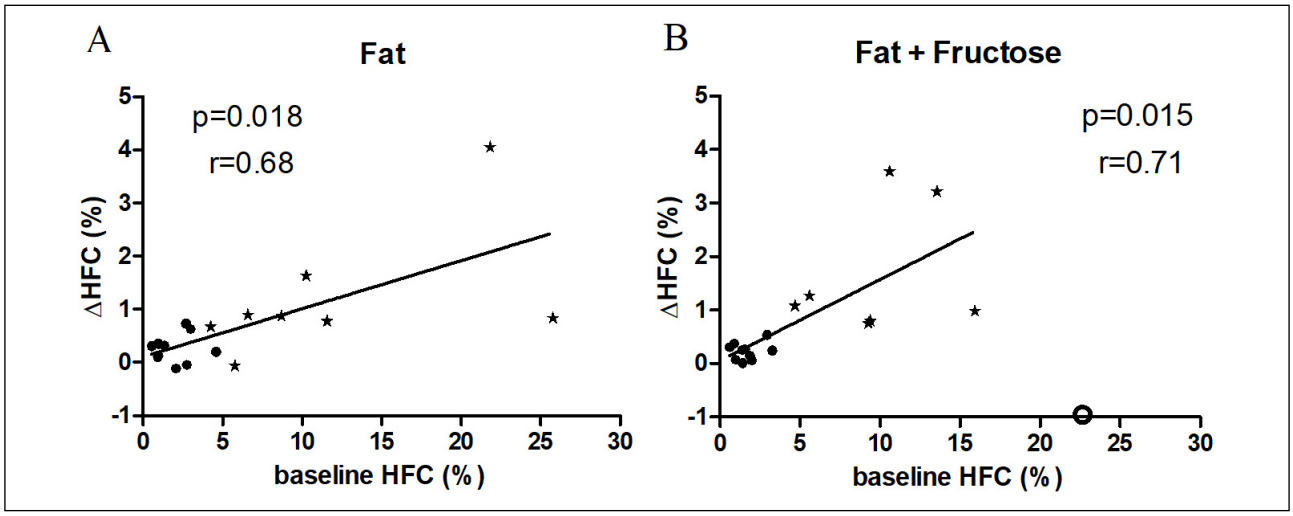

Fig. 5. Correlation of baseline (fasting) HFC and the change in HFC between time 0 and 6 hours of the experiment (ΔHFC) after fat administration (A) and fat and fructose co-administration (B). Individual values of 8 subjects with NAFLD (stars) and 10 healthy volunteers (dots) (8). One of the subjects with liver steatosis (empty circle) was excluded from analysis in B (see text). HFC, hepatic fat content.

variability of 19.7 ± 9.9%. The variability does not differ from that found in 10 healthy subjects with a normal fat content in our previous study (17.1 ± 6.7%, unpublished data) (8).

Comparison of hepatic fat content response to dietary intervention to healthy subjects

As the steatotic subjects included in the present study did not differ in their anthropometric and selected biochemical characteristics from healthy subjects in our previous study (8) and as both studies used the same design, we were able to compare the response of liver fat to different dietary interventions between both groups. Using mmANOVA, no differences in HFC response to fasting and repeated loads of glucose or fructose alone were detected between steatotic and healthy subjects. However, the response of HFC to a high-fat load was much more pronounced in steatotic subjects (from 11.8 to 13.1% at 6 hours) than in healthy subjects (from 2.0 to 2.2% at 6 hours) (8) in Fat experiment $p = 0.002$ (mmANOVA). A similar difference was found in Fat + Fructose experiment, in which HFC rose from 11.5 to 12.8% at 6 hours in steatotic subjects and from 1.7 to 1.9% at 6 hours in healthy subjects (8) ($p = 0.038$, mmANOVA). Moreover, due to the transient increase in HFC in steatotic subjects (not observed in healthy subjects), the dynamics of the changes in Fat + Glucose experiment also differed between both groups ($p < 0.001$, mmANOVA), although HFC at 6 hours did not differ from baseline in both groups.

After pooling our data from both steatotic and healthy subjects, we also analyzed the relationship between baseline HFC and increment of HFC after 6 hours in Fat and Fat + Fructose experiments, the only experiments in which HFC rose. There was a strong positive correlation between baseline HFC and HFC increment at 6 hours in Fat experiment ($r = 0.68$, $p = 0.0018$) (*Fig. 5A*) whereas no correlation was found in Fat + Fructose experiment. However, when analyzing our data in detail, we noted that HFC in this experiment rose in all but one subject with the highest baseline HFC. This subject had markedly increased his physical activity a few days before the experiment - his total energy expenditure on those days was approx. 25% higher and he had the lowest baseline HFC (22.7 versus 26.3 ± 2.1% in the other experiments) and TG (1.1 versus 2.1 ± 0.5 mmol/L in the other experiments). After exclusion of the above subject from the analysis, a strong relationship between baseline HFC and its increment after 6 hours was also found ($r = 0.71$, $p = 0.0015$) (*Fig. 5B*).

DISCUSSION

This study was focused on the immediate effects of fat, glucose, fructose and fat with glucose or fructose consumption on liver fat content in subjects with NAFLD. We found that HFC increases only in experiments with a high-fat load and that fat accumulation after a high-fat load can be reversed by glucose, but not fructose co-administration (*Fig. 4*). Administration of repeated doses of glucose or fructose alone had no effect on HFC. Importantly, as our subjects were of the same age and BMI as those with normal HFC in our previous study (8), we were able to compare the response of both groups to find out that NAFLD patients accumulate severalfold more fat in their livers after the same fat load.

However, it should be stressed that the study was not designed to study the effects of isocaloric interventions with different nutrients. The fat load alone (1460 kcal) represented approximately two thirds of daily caloric intake of our subjects whereas repeated loads of sugars carried only 600 kcal. The study thus addresses the question whether co-administration of sugars, that can induce specific metabolic responses, affects the accumulation of fat in the liver.

Hepatic fat comes from three principal sources: dietary fat, NEFA released into the circulation from adipose tissue, and DNL. The accumulation of fat in the liver after a high-fat load can be easily explained by increased fluxes of dietary fat (as TG of remnants and spillover NEFA) and NEFA originating in adipose tissue, because adipose tissue lipolysis is not efficiently inhibited by insulin (when cream containing only a small amount of lactose is given to subjects). The capacity of the liver to oxidize fatty acids or export TG in VLDL is not sufficient to compensate for the increased influx and thus fat accumulates in the liver.

Importantly, assuming a liver weight of 1.5 kg, it can be estimated that the subjects in our study accumulated approximately 18 g of fat 6 hours after consumption of 150 g of fat. Healthy subjects with a normal fat content in our previous study (8) accumulated only around 4 grams of fat in their livers in the same experiment. The design of our study does not allow us to conclusively identify the cause of the almost fivefold higher accumulation of liver fat in subjects with NAFLD. Although all subjects consumed the same amount of fat, plasma TG and NEFA concentrations (reflecting the contribution of dietary fat and adipose tissue lipolysis) increased more in steatotic subjects than in those without steatosis (TG AUIC: 8.95 ± 3.15 versus 5.40 ± 2.22 mmol/L/h, $p = 0.016$; NEFA AUIC: 1.86 ± 0.54 versus 1.08 ± 0.68 mmol/L/h, $p = 0.017$). Fat acquisition into the liver is thus increased in subjects with steatosis. In addition, it has been demonstrated that a steatotic liver loses the ability to efficiently export acquired fat. Secretion of VLDL correlates positively with HFC in healthy subjects but the correlation disappears and VLDL secretion may even decrease in subjects with an HFC higher than 5.6% (12). While unable to determine the contribution of VLDL and chylomicrons to triglyceridemia, we can assume that VLDL secretion was not increased adequately to the increased fat acquisition in our NAFLD subjects. This notion may be further supported by the relatively high frequency (0.44) of minor alleles of the *PNPLA3* and *TM6SF2* genes in steatotic subjects. These gene variants limit the availability of hepatic TG for secretion in VLDL (13, 14). In our study in non-steatotic subjects, only two subjects out of 10 (allelic frequency 0.10) carried these minor alleles (unpublished data) (8). Therefore, both the increased influx of fatty acids into the liver and lower VLDL-TG secretion can explain the several times higher fat accumulation in steatotic liver after fat consumption.

Moreover, when analyzing the data from both of our studies together, a close correlation between HFC and the change in HFC after 6 hours was found ($r = 0.6831$, $p = 0.0018$, *Fig. 5A*) suggesting that steatosis may develop in subjects predisposed to store more fat in the liver after consumption of a fatty meal.

Contrary to our findings in Fat experiment, co-administration of three 50 g doses of glucose with dairy cream at a two-hour interval (Fat + Glucose experiment) resulted in only a transient increase in HFC at 3 hours and no fat had accumulated in the liver 6 hours after cream consumption. Such a result is in an agreement with the findings of our study in healthy subjects who did not accumulate fat in their livers in the same experiment.

The fact that fat does not accumulate in the liver 6 hours after fat and glucose consumption can be explained by induction of insulin secretion and subsequent inhibition of lipolysis in adipose tissue resulting in suppression of NEFA flux from adipose tissue to the liver. This was documented in our study by no change in NEFA concentrations - the suppressive effect of insulin on NEFA concentrations (as observed after the administration of glucose alone) was likely compensated by contribution of spillover NEFA, which kept the NEFA AUIC

unchanged. Increased supply of NEFA as the principal source of hepatic fat (5) is then prevented by glucose co-administration in this experiment. Although DNL in the liver should be stimulated by insulin in Fat + Glucose experiment, it does not seem to be involved as the bulk of glucose, a potential substrate for DNL, is metabolized in extrahepatic tissues (15).

Administration of glucose alone (Glucose experiment) had no effect on HFC in our subjects whereas, in healthy subjects, HFC decreased in the same experiment (8). Nevertheless, mmANOVA did not detect a significant difference between the responses of both groups to glucose administration. However, due to the size of both groups, our study has very limited power to address questions about the role of glucose in the prevention of hepatic fat accumulation.

When three 50 g doses of fructose were co-administered with fat at two-hour intervals, the fat accumulated in the liver at 6 hours after cream consumption. The increase in HFC was even more pronounced than in Fat experiment. This was despite the fact that, same as in Fat + Glucose experiment, NEFA concentrations did not change during the experiment. The only reasonable explanation for the increase in HFC appears to be de novo lipogenesis - the bulk of fructose enters directly the liver and can be used as a substrate for DNL. Moreover, it has been repeatedly documented that fructose administration is a very efficient inducer of DNL (15-17) and that DNL is an important source of liver fat in NAFLD (5, 6).

When fructose alone was administered to subjects, the response of biochemical parameters and HFC did not differ from that in Glucose experiment. The potential stimulating effect of fructose on DNL is therefore likely not sufficient to increase HFC if fat is not present as another source of energy. However, we cannot rule out the possibility that other nutrients (such as complex carbohydrates or free glucose) providing large excess energy could have the same effect on fat accumulation in the liver.

Importantly, the suppression of lipolysis (seen as changes in NEFA concentration) did not differ between experiments with glucose and fructose administration/co-administration. That is rather surprising because fructose consumption induces less insulin response than glucose. It is not clear whether limited response of insulin to fructose administration can fully explain the suppression of lipolysis in adipose tissue. Apart of insulin action, other mechanisms may be thus involved. It has been demonstrated that fructose is stronger inducer of FGF21 in the liver than glucose (18) and FGF21 suppresses lipolysis in adipose tissue (19).

Importantly, similar to Fat experiment, the HFC response was several fold higher in subjects with NAFLD in Fat + Fructose experiment and, when analyzing pooled data from both of our studies, the increment in HFC at 6 hours correlated positively with baseline HFC ($r = 0.7068$, $p = 0.0015$) (*Fig. 5B*). However, this correlation was only achieved after the exclusion of one subject with markedly increased physical activity on days preceding the experiment as explained in the Results section.

A certain limitation of our study is the small number of subjects. This is due to the unique and complex design of our study and to difficulties with recruiting subjects with steatosis who are non-obese and non-diabetic and actually not typical NAFLD patients. However, we chose to select such patients to eliminate the impact of overt insulin resistance on metabolic pathways directly involved in hepatic fat accumulation. On the other hand, although our NAFLD patients had the same anthropometric characteristics and the same plasma glucose and NEFA concentrations as BMI- and age-matched healthy subjects in our previous study (8), they were found to have slightly increased insulinemia (albeit still within the normal range). This may suggest that they need to secrete more insulin to keep their glycemia and lipolysis under control and, hence, that they are in the initial stage of developing insulin resistance. As our subjects had already markedly developed steatosis, our findings may support the notion that NAFLD is not a simple component of metabolic syndrome but, rather, one of the first components or even the precursor of metabolic syndrome as proposed by others (20). Another limitation of the study is that only male subjects were studied. However, the inclusion of both sexes in such a small group would complicate the interpretation of the study results because of potential impact of cycle and menopause on regulation of hepatic metabolism in women.

The design of our study allowed us to investigate the direct impact of particular nutrients on immediate quantitative changes in hepatic fat content. The results clearly highlight the important role played by dietary fat in the pathogenesis of steatosis. Although the fat load used in our study was very high, one can assume that the smaller doses of fat consumed in real life will lead to similar changes not detectable by MRS but, possibly, cumulative in the long run.

More importantly, our data clearly point out the important difference between glucose and fructose. Increased fructose consumption is believed to be an important factor in the development of obesity and NAFLD in the general population (21, 22); however, it is still a subject of debate whether or not fructose is directly involved in the pathogenesis of NAFLD or whether it should be viewed upon as just a marker of increased caloric intake (23, 24). Our data clearly implicate fructose as a culprit directly involved in pathogenesis of hepatosteatosis, especially if associated with a high fat intake.

Moreover, the use of repeated administration of simple sugars represents a major strength of our study mainly because the effect of a simple bolus of glucose on insulin-induced suppression of lipolysis is only short-term and plasma NEFA concentrations return to baseline rather quickly. As only repeated doses of glucose can keep NEFA concentrations suppressed throughout the experiment, this strategy was adopted to study the effect of a limited NEFA input on HFC. Importantly, such an experimental design may not be far from real life - it is comparable to drinking 1.5 L of a soft drink within six hours.

Last but not least, the design of our study allowed us to determine the intra-individual variability of HFC being slightly below 20%. Therefore, if taking intra-individual variability into account, the diagnosis of hepatosteatosis using ^{1}H-MRS should only be considered definitive when HFC exceeds the upper limit of normal values by 2 standard deviations, *i.e.* approximately at a value of 7.6% (assuming an upper limit of 5.6% (25)).

In summary, in patients with NAFLD, hepatic fat content increased 6 hours after a high-fat load or a high-fat load with repeated doses of fructose. On the contrary, when co-administering glucose with fat, HFC rose only transiently to return to baseline at 6 hours. When compared with age- and BMI-matched healthy subjects with a normal HFC (8), patients with steatosis accumulate severalfold more fat in their livers after the same load of dietary fat. No change in HFC was observed during fasting or after repeated doses of glucose or fructose.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUIC, area under the increment curve; CRP, C-reactive protein; DNL, de novo lipogenesis; GGT, gamma-glutamyltransferase; ^{1}H-MRS, proton magnetic resonance spectroscopy; HFC, hepatic fat content; mm ANOVA, mixed model ANOVA for repeated measures with one grouping factor - steatosis; NAFLD, non-alcoholic fatty liver disease; NEFA, non-esterified fatty acids; TG, triglycerides; VLDL, very low-density lipoprotein.

Authors' contribution: J. Kovar and T. Dusilova contributed equally to the project.

Acknowledgments: We thank Jolana Mrazkova, Romana Houdkova, Regina Stupalova, Nora Stankova and Petr Stavek for their excellent technical assistance, Magdalena Neroldova and Lucie Budisova for genetic analysis, and Vera Lanska for her help with statistical analysis of data.

Funding: This work was supported by the Ministry of Health of the Czech Republic, grant nr. 16-28427A and by the Ministry of Health of the Czech Republic - DRO ("Institute for Clinical and Experimental Medicine - IKEM, IN 00023001"). All rights reserved.

Conflict of interests: None declared.

REFERENCES

1. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011; 34: 274-285.
2. Allen AM, Therneau TM, Larson JJ, Coward A, Somers VK, Kamath PS. Nonalcoholic fatty liver disease incidence and impact on metabolic burden and death: a 20 year-community study. *Hepatology* 2018; 67: 1726-1736.
3. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. *Hepatology* 2018; 67: 123-133.
4. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; 332: 1519-1523.
5. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005; 115: 1343-1351.
6. Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology* 2014; 146: 726-735.
7. Miele L, Zocco MA, Pizzolante F, *et al.* Use of imaging techniques for non-invasive assessment in the diagnosis and staging of non-alcoholic fatty liver disease. *Metabolism* 2020; 112: 154355. doi: 10.1016/j.metabol.2020.154355
8. Dusilova T, Kovar J, Drobny M, *et al.* Different acute effects of fructose and glucose administration on hepatic fat content. *Am J Clin Nutr* 2019; 109: 1519-1526.
9. Chalasani N, Younossi Z, Lavine JE, *et al.* The diagnosis and management of nonalcoholic fatty liver disease: practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; 67: 328-357.
10. Hajek M, Dezortova M, Wagnerova D, *et al.* MR spectroscopy as a tool for in vivo determination of steatosis in liver transplant recipients. *MAGMA* 2011; 24: 297-304.
11. Longo R, Pollesello P, Ricci C, *et al.* Proton MR spectroscopy in quantitative in vivo determination of fat content in human liver steatosis. *J Magn Reson Imaging* 1995; 5: 281-285.
12. Lytle KA, Bush NC, Triay JM, *et al.* Hepatic fatty acid balance and hepatic fat content in humans with severe obesity. *J Clin Endocrinol Metab* 2019; 104: 6171-6181.
13. Wang Y, Kory N, BasuRay S, Cohen JC, Hobbs HH. PNPLA3, CGI-58, and inhibition of hepatic triglyceride hydrolysis in mice. *Hepatology* 2019; 69: 2427-2441.
14. Smagris E, Gilyard S, BasuRay S, Cohen JC, Hobbs HH. Inactivation of Tm6sf2, a gene defective in fatty liver disease, impairs lipidation but not secretion of very low density lipoproteins. *J Biol Chem* 2016; 291: 10659-10676.
15. Lustig RH. Fructose: metabolic, hedonic, and societal parallels with ethanol. *J Am Diet Assoc* 2010; 110: 1307-1321.
16. Geidl-Flueck B, Gerber PA. Insights into the hexose liver metabolism-glucose versus fructose. *Nutrients* 2017; 9: 1026. doi: 10.3390/nu9091026
17. Ter Horst KW, Serlie MJ. Fructose consumption, lipogenesis, and non-alcoholic fatty liver disease. *Nutrients* 2017; 9: 891. doi: 10.3390/nu9090981
18. Dushay JR, Toschi E, Mitten EK, Fisher FM, Herman MA, Maratos-Flier E. Fructose ingestion acutely stimulates circulating FGF21 levels in humans. *Mol Metab* 2015; 4: 51-57.
19. Park JG, Xu X, Cho S, *et al.* CREBH-FGF21 axis improves hepatic steatosis by suppressing adipose tissue lipolysis. *Sci Rep* 2016; 6: 27938. doi: 10.1038/srep27938
20. Lonardo A, Ballestri S, Marchesini G, Angulo P, Loria P. Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. *Dig Liver Dis* 2015; 47: 181-190.
21. Stanhope KL, Schwarz JM, Havel PJ. Adverse metabolic effects of dietary fructose: results from the recent epidemiological, clinical, and mechanistic studies. *Curr Opin Lipidol* 2013; 24: 198-206.
22. Bray GA, Popkin BM. Dietary sugar and body weight: have we reached a crisis in the epidemic of obesity and diabetes? Health be damned! Pour on the sugar. *Diabetes Care* 2014; 37: 950-956.
23. Khan TA, Sievenpiper JL. Controversies about sugars: results from systematic reviews and meta-analyses on obesity, cardiometabolic disease and diabetes. *Eur J Nutr* 2016; 55 (Suppl. 2): 25-43.
24. Stanhope KL. Sugar consumption, metabolic disease and obesity: the state of the controversy. *Crit Rev Clin Lab Sci* 2016; 53: 52-67.
25. Szczepaniak LS, Nurenberg P, Leonard D, *et al.* Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; 288: E462-E468.

Received: January 17, 2021
Accepted: February 26, 2021

Author's address: Jan Kovar, Institute for Clinical and Experimental Medicine, Videnska 1958/9, 140 21 Prague 4, Czech Republic.
E-mail: jan.kovar@ikem.cz