# Chronic fructose intake does not induce liver steatosis and inflammation in female Sprague–Dawley rats, but causes hypertriglyceridemia related to decreased VLDL receptor expression

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## Abstract

### Purpose

Sugar-sweetened beverage intake is a risk factor for insulin resistance, dyslipidemia, fatty liver, and steatohepatitis (NASH). Sub-chronic supplementation of liquid fructose, but not glucose, in female rats increases liver and plasma triglycerides without inflammation. We hypothesized that chronic supplementation of fructose would cause NASH and liver insulin resistance.

**AQ1**

## Methods

We supplemented female Sprague–Dawley rats with water or either fructose or glucose 10% w/v solutions under isocaloric conditions for 7 months. At the end, plasma analytes, insulin, and adiponectin were determined, as well as liver triglyceride content and the expression of key genes controlling inflammation, fatty acid synthesis and oxidation, endoplasmic reticulum stress, and plasma VLDL clearance, by biochemical and histological methods.

**AQ2**

## Results

Although sugar-supplemented rats increased their energy intake by 50–60%, we found no manifestation of liver steatosis, fibrosis or necrosis, unchanged plasma or tissue markers of inflammation or fibrosis, and reduced liver expression of gluconeogenic enzymes, despite both sugars increased fatty acid synthesis, mTORC1, and IRE1 activity, while decreasing fatty acid oxidation and PPARα activity. Only fructose-supplemented rats were hypertriglyceridemic, showing a reduced expression of VLDL receptor and lipoprotein lipase in skeletal muscle and vWAT. Glucose-supplemented rats showed increased adiponectinemia, which would explain the different metabolic outcomes of the two sugars.

## Conclusions

Chronic liquid simple sugar supplementation, as the sole risk factor, is not enough for female rats to develop NASH and increased liver gluconeogenesis. Nevertheless, under isocaloric conditions, only fructose induced hypertriglyceridemia, thus confirming that also the type of nutrient matters in the development of metabolic diseases.

## Keywords

Glucose
NAFLD
Fatty acid metabolism
Endoplasmic reticulum stress
UPR response

Marta Alegret and Juan Carlos Laguna contributed equally to this work.

Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00394-018-1654-9) contains supplementary material, which is available to authorized users.

# Introduction

Epidemiological and intervention studies in humans [1, 2], as well as data generated from experimental animals point to the unrestricted consumption of simple sugars, especially fructose, as a risk factor for the development of metabolic disorders, such as obesity, insulin resistance, dyslipidemia, and non-alcoholic fatty liver disease (NAFLD) [3, 4]. There is a continuous academic debate over whether these sugar-related metabolic toxicities are due to the high caloric burden provided by simple sugars or, on top of it, to a particular metabolic characteristic of fructose [5].

We have previously shown that sub-chronic supplementation (2 months) in female Sprague–Dawley rats with liquid fructose (10% w/v), but not glucose with similar calorie intake, impairs insulin signaling in liver, skeletal muscle and white adipose tissue [6]. Nevertheless, despite inducing hypertriglyceridemia, eliciting a reduction in insulin receptor substrate 2 (IRS2) and V-akt murine thymoma viral oncogene homolog-2 (Akt) phosphorylation [7], in addition to steatosis [8] in the liver, fructose supplementation did not clearly increase the expression of hepatic inflammatory markers and gluconeogenic enzymes, the latter of which is a key manifestation of insulin resistance in hepatic tissue that results in sustained gluconeogenesis [9].

Humans who habitually consume sugar-sweetened beverages do so for a considerable proportion of their lifetime, often more than 10 years, and this practice has been associated with a high incidence of Type 2 Diabetes (T2D) [10]. Fructose consumption in humans has also been directly linked to the development

of NAFLD [4, 11, 12]. Furthermore, NAFLD increases the risk of death among diabetic patients [13], and includes an overlapping continuum of progressive liver-metabolism deterioration from asymptomatic hepatic steatosis, with a reduced but significant proportion of patients progressing to non-alcoholic steatohepatitis or NASH, fibrotic/cirrhotic states and development of hepatocellular carcinoma over time [14, 15].

In our previous studies, young adult female Sprague–Dawley rats were subjected to continuous liquid simple sugar supplementation from 15 days to 2 months [6, 7, 8, 16]. In these studies, we showed that female rats supplemented for short periods of time (14 days) with liquid fructose displayed a more detrimental response than male rats. Specifically, we reported that fructose induced hypertriglyceridemia and fatty liver in both sexes, but only females showed glucose intolerance and hepatic insulin resistance. Furthermore, accretion of liver triglycerides up to 2 months continuous fructose supplementation was confirmed by Oil-Red O histology and quantitation of liver tissue triglyceride concentration. Considering that one rat month is roughly equivalent to three human years [17], our previous sub-acute and sub-chronic experimental studies correspond to continuous human exposures to sugar-sweetened beverages for 1–6 years. Thus, we wondered whether a chronic time frame of continuous liquid simple sugar supplementation in female rats, equivalent to 15–20 years of human life, would be enough for them to develop clear liver insulin resistance with increased expression of gluconeogenic enzymes and fatty liver with significant manifestations of an inflammatory process or NASH. We have used simple sugar supplementation at a 10% weight/volume concentration for at least three reasons: (1) to be consistent with our previous studies of short periods of administration and allow direct comparison of results; (2) to mimic the most usual pattern of simple sugar consumption in human populations, as sugar-sweetened beverages; and (3) to minimize possible direct effects on intestinal mucosae favoring the development of intestinal fructose intolerance, increased permeability of the gut barrier and consequent leaking of enterobacterial toxins to the portal blood, that are recognized risk factors for the development of steatohepatitis.

In the present work, we show that female Sprague–Dawley rats, supplemented with either fructose or glucose liquid solutions (10% w/v) for 7 months, increased their energy intake by 50–60% (with both supplemented sugars providing the same numbers of calories), but did not show any clear manifestation of steatohepatitis and even significantly reduced their liver expression of gluconeogenic enzymes, glucose-6-phosphatase (G6Pc) and phosphoenolpyruvate

carboxykinase (PEPCK). Nevertheless, only fructose induced clear hypertriglyceridemia, which was probably related to both the reduced fatty acid catabolism of the liver and peripheral very low-density lipoprotein (VLDL) clearance.

# Materials and methods

## Animals and diets

Female Sprague–Dawley rats obtained from Charles River (Barcelona, Spain) were used for all experiments. Rats were maintained under conditions of constant humidity (40–60%) and temperature (20–24 °C) with a light/dark cycle of 12 h. Procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee (Autonomous Government of Catalonia Act 5/1995 of 21 July). All experimental procedures involving animals were approved by the University of Barcelona's Animal Experimentation Ethics Committee (approval no. 7912).

Twenty-four rats aged 8 weeks were randomly assigned to either a control group (no supplementary sugar, $n = 8$), a fructose-supplemented group (10% w/v in drinking water, $n = 8$) or a group supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group for 7 months ($n = 8$), as described in [6].

## Sample preparation

At the end of intervention, after a 12-h fast, rats from each group were anesthetized with ketamine/xylazine (9-mg and 40-µg/100 g body weight, respectively) and blood samples were obtained by intracardiac puncture and collected in micro tubes (Sarstedt AG & Co., Nümbrecht, Germany). Plasma was prepared by centrifugation at 3000×*g* for 10 min at room temperature and stored at − 80 °C until used. Rats were euthanized by exsanguination, and samples from liver, visceral white adipose tissue (vWAT) and gastrocnemius muscle from both legs were collected, immediately frozen in liquid nitrogen, and stored at − 80 °C until needed.

## Blood and plasma parameters

Fasting triglyceride, cholesterol, and glucose levels were measured using an Accutrend® Plus System glucometer (Cobas, Roche Farma, Barcelona, Spain) in blood samples collected from the tail vein. Plasma insulin and adiponectin

concentrations were determined using specific enzyme-linked immunosorbent assay kits (Millipore, Billerica, MA, USA). Alanine aminotransferase (ALT) activity was determined using an ALT/GPT enzymatic kit (Spinreact, Girona, Spain).

## Liver assays

Liver triglycerides were extracted as described by Qu et al. [18] and determined using a triglyceride colorimetric kit (Spinreact, Girona, Spain). Total hepatic fatty acid β-oxidation was determined in rat livers as described by Lazarow [19], using 30 μg of postnuclear supernatant.

## RNA preparation and analysis

Total RNA was isolated with the Trizol® Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. RNA concentration and purity were measured spectrophotometrically using the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). The ratios of absorbance at 260/230 and at 260/280 were used as indicators of RNA purity. cDNA was synthesized by reverse transcription as described by Hutter et al. [20]. Specific mRNAs were assessed by real-time reverse transcription polymerase chain reaction (RT-PCR) using SYBR green PCR Master Mix, specific primers and the Applied Biosystems StepOne-Plus sequence detection system (Applied Biosystems, Foster City, CA, USA). β-actin (*actb*) was used as an internal control. Primer sequences and PCR product length are listed in Supplementary Table 1.

## Preparation of protein extracts

Liver and muscle samples were homogenized with a Dounce homogenizer (BCD-6015, Caframo) and a mechanic homogenizer (Polytron® PT 1200E), respectively. vWAT samples were micronized by freezing them with liquid nitrogen and grinding them with a mortar. For total protein extraction, samples were homogenized with a lysis buffer with proteases, phosphatases and deacetylases inhibitors, and incubated for 1.5 h at 4 °C. Samples were then centrifuged at 15,000×*g* for 15 min at 4 °C and the supernatants collected. To obtain hepatic nuclear extracts, samples were homogenized with a homogenization buffer, kept on ice for 10 min and centrifuged at 1000×*g* for 10 min at 4 °C. Lysis buffer was added to the pellet that was obtained and samples were incubated for 1.5 h at 4 °C, centrifuged at 25,000×*g* for 30 min at 4 °C, and the supernatants collected. The composition of the buffers was as described by Sangüesa et al. [21]. Protein concentrations were determined by the

Bradford method [22].

## Western blot analysis

Different protein extracts (20–30 μg) from rat tissues were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA, USA), blocked for 1 h at room temperature with 5% non-fat milk solution in Tris-buffered saline (TBS) with 0.1% Tween-20, and incubated as described previously [23]. Detection was performed using the Immobilion Western HRP substrate Peroxide Solution® (Millipore, Billerica, MA). To confirm the uniformity of protein loading, blots were incubated with β-actin or β-tubulin antibody (Sigma–Aldrich, St. Louis, MO, USA) as a control. Primary antibodies for phosphor-4EBP1 (#2855), phosphor- (#3661) and total- (#3662) ACC, phosphor- (#2535) and total- (#2532) AMPKα, phosphor- (Ser473) (#9271), phosphor- (Thr308) (#9275) and total- (#9272) Akt, phosphor- (#9101) ERK1/2, phosphor- (#9336) and total- (#9315) GSK3β, phosphor- (#9251) and total- (#9252) JNK, and mTOR (#2972) were supplied by Cell Signaling (Danvers, MA, USA). Phosphor-mTOR was purchased from Millipore (Billerica, MA, USA). ATF6α (sc-22799), ChREBP (sc-21189), total-4EBP1 (sc-9977), FAS (sc-55580), FK (sc-50029), phosphor- (sc-11350) and total- (sc-11350) FoxO1, G6Pc (sc-33839), IRS2 (sc-1555), MTP (sc-135994), PEPCK (sc-74823), PGC1α (sc-5816), phosphor- (sc-32577) and total- (sc-13073) PERK, SREBP-1 (sc-366) and VLDLR (sc-18824) were obtained from Santa Cruz Biotechnologies (Dallas, TX, USA). Phosphor- (ab37037) and total- (ab104157) IRE1, LPL (ab93898), PPARα (ab8934) and SCD1 (ab19862) antibodies were obtained from AbCam (Cambridge, UK). Phosphor-PGC1α (S571) was purchased from RD Systems (Minneapolis, MN, USA). Antibody against rat liver CPT-IA was kindly provided by L. Herrero and D. Serra (Department of Biochemistry and Physiology, School of Pharmacy and Food Sciences, University of Barcelona).

## Histological studies

Paraffin-embedded liver sections were stained with hematoxylin and eosin and with Masson's trichrome acid to assess the degree of necrosis and fibrosis, respectively. A pathologist blinded to the treatment groups performed the histological analysis at BioBanc (Banc de tumors-IDIBAPS, Barcelona, Spain). Necrosis was scored as 0 (absent), 1 (< 1%), 2 (< 5%), 3 (< 10%), or 4 (≥ 10%). Fibrosis was categorized as 0 (no fibrosis), 1 (portal or sinusoidal fibrosis without septa), 2 (portal or sinusoidal fibrosis with rare septa), 3 (abundant septa without

cirrhosis), or 4 (cirrhosis). Lipid accumulation was analyzed in the liver sections stained with Oil-Red O. Images were acquired with an Olympus BX51 microscope and analyzed using Image the J 1.49v software (National Institutes of Health, USA). The area of positive staining for Oil-Red O was calculated as a percentage of stained area/total section area in each sample. Steatosis was categorized as 0 (< 5%), 1 (5–10%), 2 (10–30%), and 3 (> 30%).

### Statistical analysis

The results are expressed as the mean of 8 values ± standard deviation (SD), unless otherwise stated. Plasma samples were assayed in duplicate. The Gaussian distribution of the data was verified using the Kolmogorov–Smirnov normality test, and significant differences were established by one-way ANOVA and Šidák's post-hoc test for selected comparisons (GraphPad Software V6). When variance was not homogeneous, a non-parametric test was performed. The level of statistical significance was set at $P \leq 0.05$.

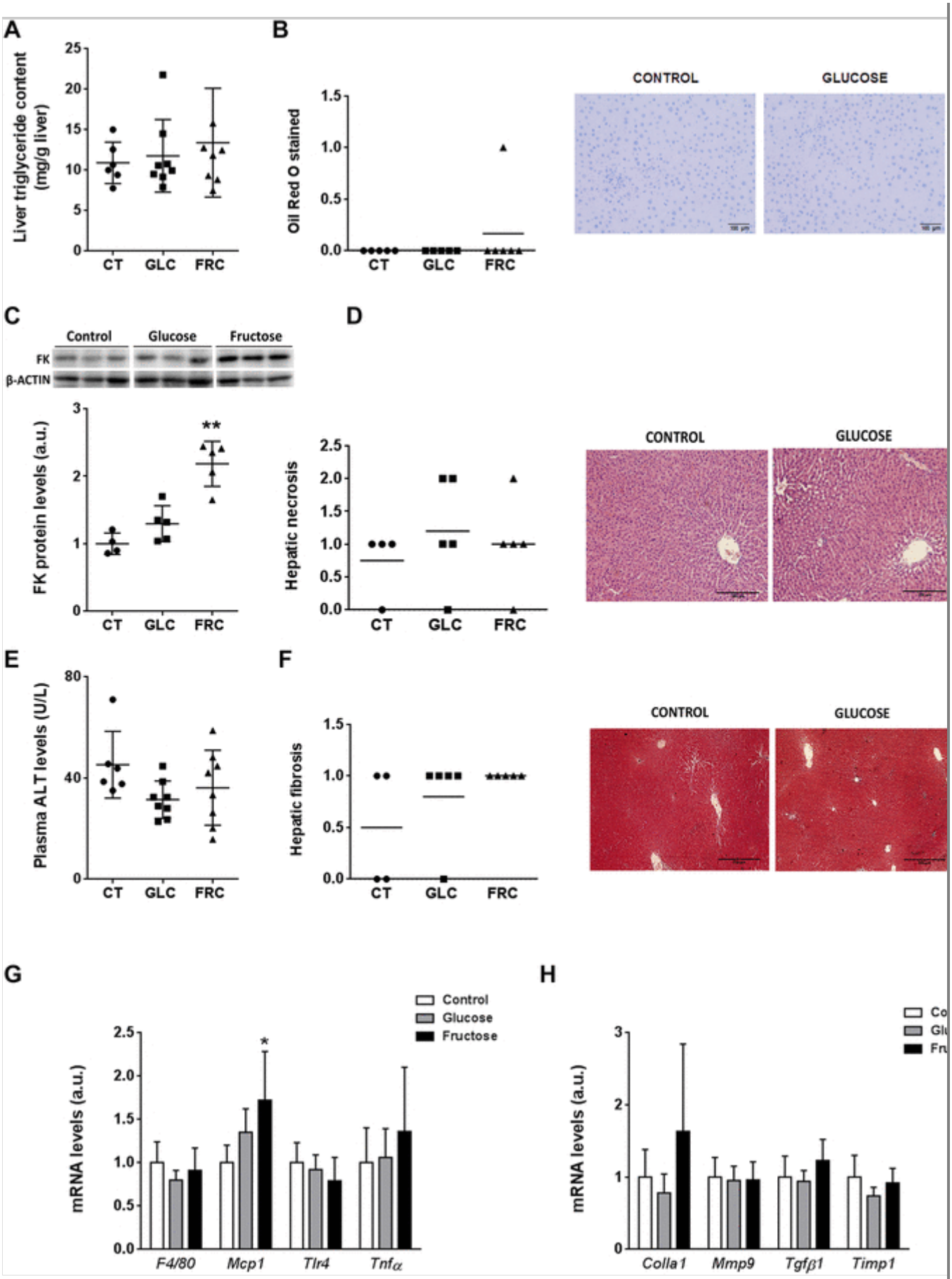
## Results

### Female rats consuming glucose or fructose showed no clear signs of NAFLD or NASH after 7 months supplementation

Zoometric parameters for these sugar-supplemented rats have been reported previously and are shown in Supplementary Table 2; total caloric intake was similarly increased in both glucose- and fructose-supplemented groups (1.6 and 1.5-fold, respectively). Neither glucose-, nor fructose-supplemented animals showed increased liver triglycerides, assessed chemically as mg of triglyceride per gram of liver (Fig. 1a), or histologically by examining Oil-Red O-stained liver samples (Fig. 1b). The lack of a clear steatotic effect was not due to deficient fructose metabolism in the liver, as the fructokinase levels increased in the livers of fructose-supplemented rats (Fig. 1c). Furthermore, neither glucose-, nor fructose-supplemented rats showed clear histological signs of necrosis or fibrosis, as assessed by Haematoxylin and Eosin stain and Masson's trichrome stain, respectively (Fig. 1d, f). Consequently, neither plasma concentrations of alanine aminotransferase (ALT) (Fig. 1e), nor hepatic mRNA levels corresponding to genes related to inflammatory (Fig. 1g) or fibrogenic (Fig. 1h) processes were significantly altered by simple sugar supplementation, except for monocyte chemoattractant protein-1 (*mcp1*) expression, that was increased 1.7-fold in the livers of fructose-supplemented rats.

**Fig. 1**

Liver triglyceride content (**a**) from CT (control female Sprague–Dawley—SD—rats), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group) and FRC (female SD rats supplemented with a 10% w/v fructose solution as drinking water for 7 months) experimental groups; scatter plots represent individual values and the mean ± SD for each group. Representative Oil-Red O (**b**) liver sections from CT, GLC, and FRC groups are shown; on the left side of the figure, scatter plots of steatosis scores from five different samples of each group are shown. **c** Western-blot of fructokinase (FK) protein in liver samples obtained from the three experimental dietary groups; scatter plots represent individual values and the mean ± SD band intensity for each group. Representative bands shown in the upper part of the figure correspond to three different rats in each group. Representative hematoxylin and eosin (**d**) liver sections from CT, GLC, and FRC groups; on the left side of the figure, scatter plots of necrosis scores from four to five different samples of each group are shown. **e** Plasma ALT concentrations from CT, GLC, and FRC groups. Scatter plots represent individual values and the mean ± SD for each group. Representative Masson's trichrome acid (**f**) liver sections from CT, GLC, and FRC groups; on the left side of the figure, scatter plots of fibrosis scores from four to five different samples of each group are shown. Bar plots represent the mean ± SD. mRNA levels corresponding to genes related to liver inflammation (**g**) and fibrosis (**h**) from CT ($n = 6$), GLC ($n = 8$), and FRC ($n = 8$) groups. *$P < 0.05$, **$P < 0.01$ vs CT values. *Mcp1* monocyte chemoattractant protein 1, *tlr4* toll-like receptor 4, *tnfα* tumor necrosis factor α, *colla1* collagen type 1, alpha I chain, *mmp9* matrix metallopeptidase 9, *tgfβ1* tumor growth factor β1, *timp1* tissue inhibitor of metalloproteinase 1



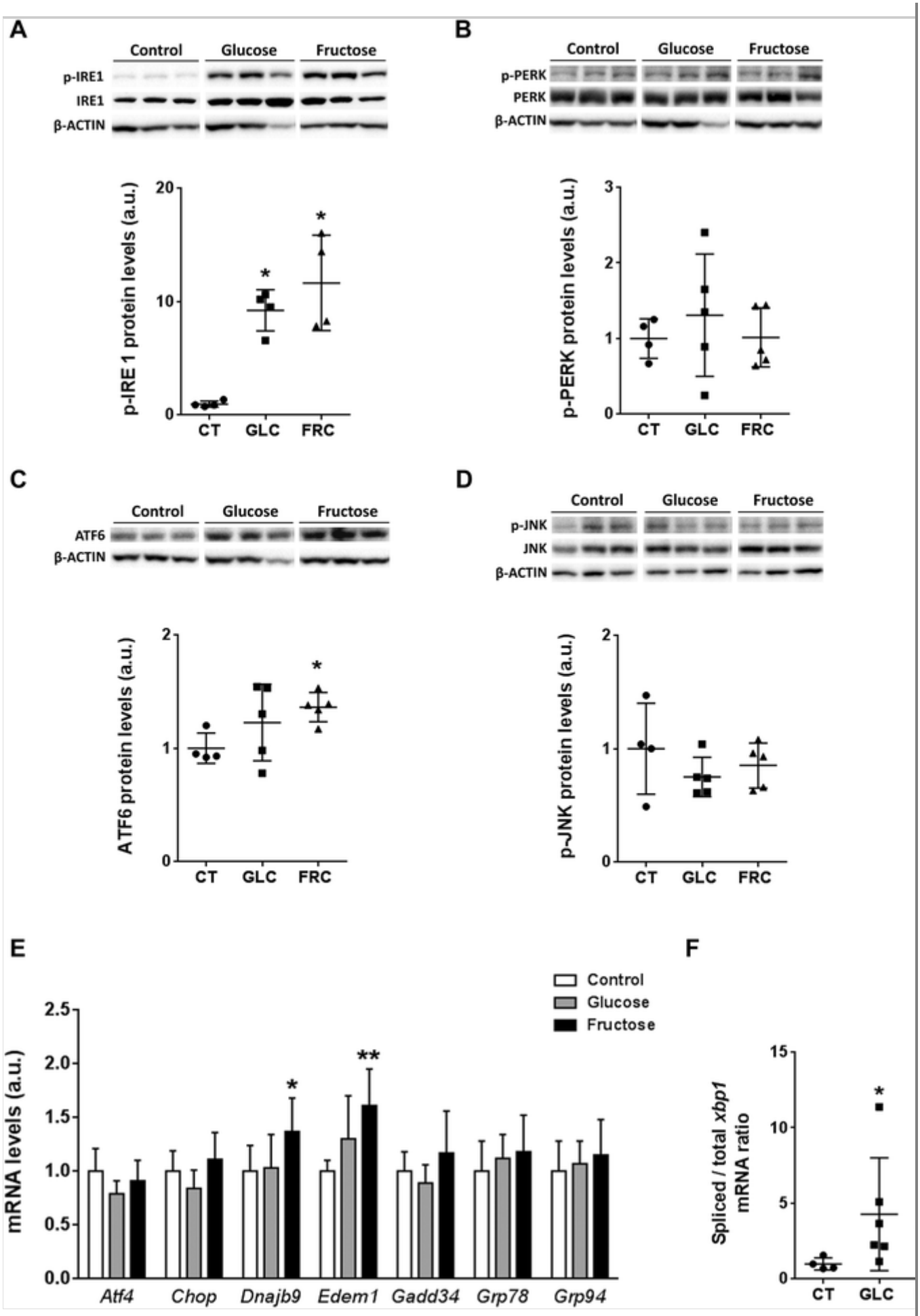

The chronic supplementation of simple sugars in female Sprague–Dawley rats significantly increased inositol requiring enzyme 1 (IRE1) phosphorylation

(Fig. 2a) did not modify the degree of activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) (Fig. 2b), and, in the livers of fructose-supplemented animals only, increased the amount of the active form of activation transcription factor 6 (ATF6) (Fig. 2c). Furthermore, there was no activation of the c-Jun N-terminal kinase (JNK), assessed by its degree of phosphorylation at Thr183/Tyr185 (Fig. 2d). This implies a lack of stimulation of the kinase activity of IRE1 [24]. Moreover, there was no increase in the expression of the majority of genes controlled by the IRE1 and PERK branches of the Unfolded Protein Response (UPR) (Fig. 2e). The only genes whose expression increased in the livers of fructose-supplemented rats were endoplasmic reticulum degradation enhancing alpha-mannosidase like protein 1 (*edem1*) and DNA J heat shock protein family (Hsp40) member B 9 (*dnajb9*), related to the endoplasmic reticulum-associated protein degradation (ERAD) system. The expression of these genes is controlled by the concerted action of the transcription factors X-box-binding protein 1 (XBP1) and ATF6 [25]. Although we did not detect XBP1 protein in our samples, probably due to the long fasting period and the short half-life of the XBP1 protein [26], we found a significant increase in the ratio between the spliced and total *xbp1* mRNA in samples from sugar-supplemented animals (Fig. 2f). This suggests an increase in IRE1-related RNAse activity in these samples.

**Fig. 2**

Western-blot of phosphor- and total-IRE1 (**a**), phosphor- and total-PERK (**b**), total-ATF6 (**c**), and phosphor- and total-JNK (**d**) proteins, respectively, in liver samples obtained from CT (control female Sprague–Dawley—SD—rats), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group), and FRC (female SD rats supplemented with a 10% w/v fructose solution as drinking water for 7 months) experimental groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity of the phosphor- or total-proteins for each group. Representative bands shown in the upper part of the figure correspond to three different rats in each group. **e** Bar plots representing the mean ± SD. mRNA levels corresponding to liver genes controlled by the IRE1 and PERK branches of the Unfolded Protein Response (UPR) from CT ($n = 6$), GLC ($n = 8$), and FRC ($n = 8$) groups. **f** Scatter plots represent individual values of four–six different samples and the mean ± SD of the ratio between spliced / total *xbp1* mRNA from CT, GLC, and FRC groups. $*P < 0.05$, $**P < 0.01$ vs CT values. *Atf4* activating transcription factor 4, *chop* C/EBP homologous protein, *dnajb9* DNA J heat shock protein family (Hsp40) member B 9, *edem1* endoplasmic reticulum degradation enhancing alpha-mannosidase like

protein 1, *gadd34* growth arrest and DNA damage-inducible protein 34, *grp78* glucose-related protein 78, and *grp94* glucose-related protein 94


A
Control
Glucose
Fructose
p-IRE1
IRE1
β-ACTIN
p-IRE 1 protein levels (a.u.)
20
10
0
CT
GLC
FRC
B
Control
Glucose
Fructose
p-PERK
PERK
β-ACTIN
p-PERK protein levels (a.u.)
3
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CT
GLC
FRC
C
Control
Glucose
Fructose
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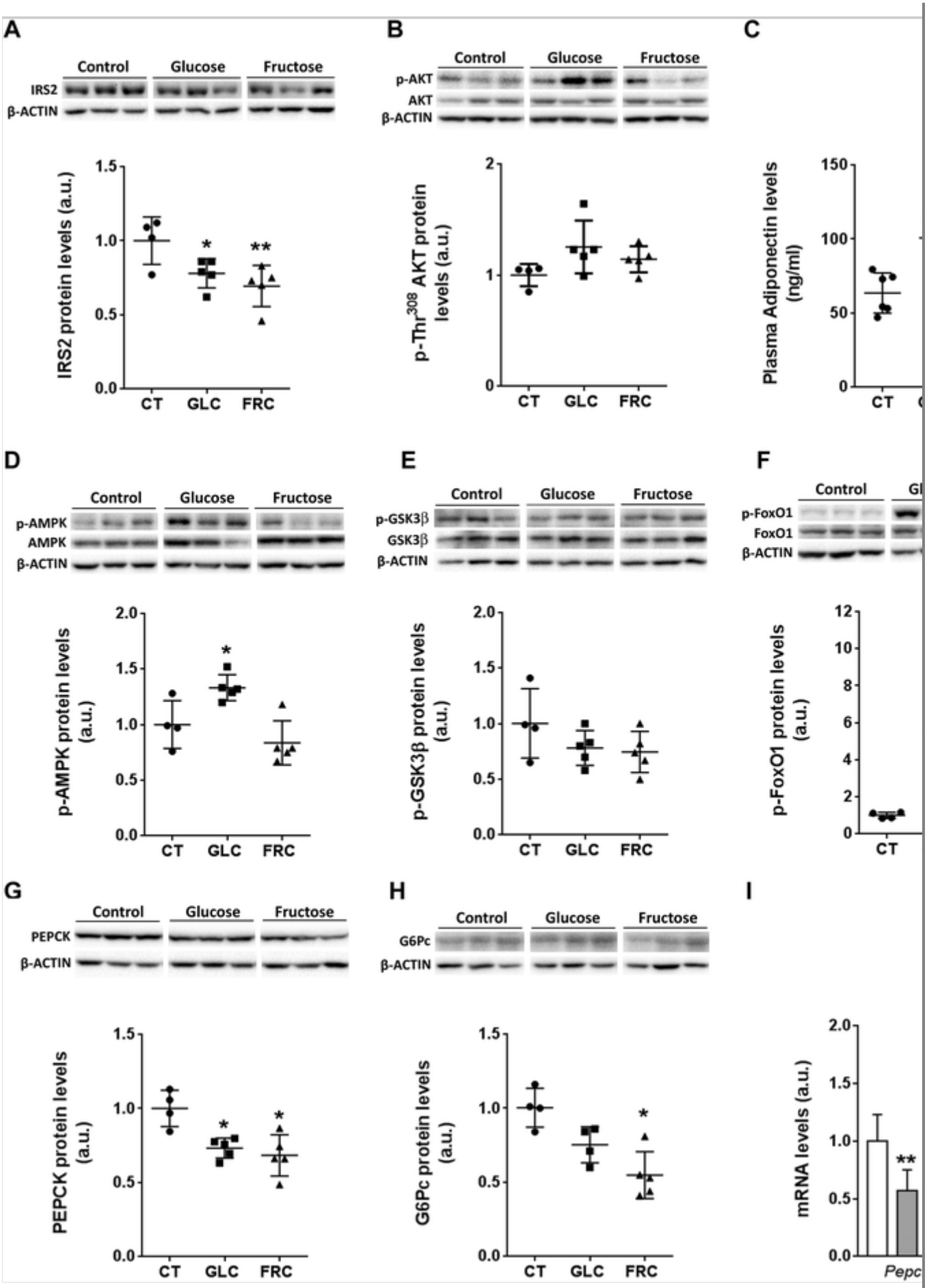
mRNA levels (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
Atf4
Chop
Dnajb9
Edem1
Gadd34
Grp78
Grp94
F
Spliced / total xbp1 mRNA ratio
15
10
5
0
CT
GLC

## Female rats consuming glucose or fructose showed no clear signs of hepatic insulin resistance, with a significant reduction in the expression of key gluconeogenic genes, after 7 months supplementation

Only fructose-supplemented animals, despite being normoglycemic, showed increased plasma insulin concentrations, and reduced insulin sensitivity index (ISI, see Supplementary Table 2). Nevertheless, the reduction in ISI values could not be attributed primarily to a hepatic origin. Despite reductions in the amount of liver insulin receptor substrate 2 (IRS2) protein (Fig. 3a), the degree of Akt phosphorylation at Thr 308, an insulin-signaling event transduced via IRS2—phosphatidylinositide 3-kinase—3-phosphoinositide dependent protein kinase-1 [27], was not modified in liver samples from fructose-supplemented rats, and even increased non-significantly in samples from glucose-supplemented rats (Fig. 3b), as a result of the mild hyperadiponectinemia (Fig. 3c) related to glucose consumption. As a marker of adiponectin activity, only liver samples from glucose-supplemented rats showed a significant increase in the phosphorylated, active form of AMP-activated protein kinase (AMPK) (Fig. 3d), a downstream effector of adiponectin signaling in liver [28].

**Fig. 3**

Western-blot of IRS2 (**a**), phosphor ($Thr^{308}$)- and total-Akt (**b**), phosphor- and total-AMPK (**d**), phosphor- and total-GSK3β (**e**), phosphor- and total-FoxO1 (**f**), PEPCK (**g**), and G6Pc (**h**) proteins, respectively, in liver samples obtained from CT (control female Sprague–Dawley—SD—rats), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group), and FRC (female SD rats supplemented with a 10% w/v fructose solution as drinking water for seven months) experimental groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity of the phosphor- (**b, d, e, f**) or total- (**a, g, h**) proteins. Representative bands shown in the upper part of the figure correspond to three different rats in each group. **c** Plasma adiponectin concentrations from CT, GLC, and FRC groups. Scatter plots represent individual values and the mean ± SD for each group. **i** Bar plots representing the mean ± SD mRNA levels corresponding to liver *pepck* and *g6pc* genes from CT ($n = 6$), GLC ($n = 8$), and FRC ($n = 8$) groups. *$P < 0.05$, **$P < 0.01$ vs CT values




With regard to the two main substrates of Akt kinase activity, the degree of

phosphorylation of glycogen synthase kinase 3 β remained unchanged (Fig. 3e), while that of forkhead box protein O1 (FoxO1) was clearly increased by both sugars (Fig. 3f). Since the unphosphorylated, active form of FoxO1 is a key factor in gluconeogenic gene transcription [29], the expression of two key gluconeogenic genes, *pepck* and *g6pc*, showed a clear reduction in the livers of sugar-supplemented animals (Fig. 3g, h, i), in spite of an unmodified transduction of insulin signaling from receptor to the phosphorylation event of Akt.

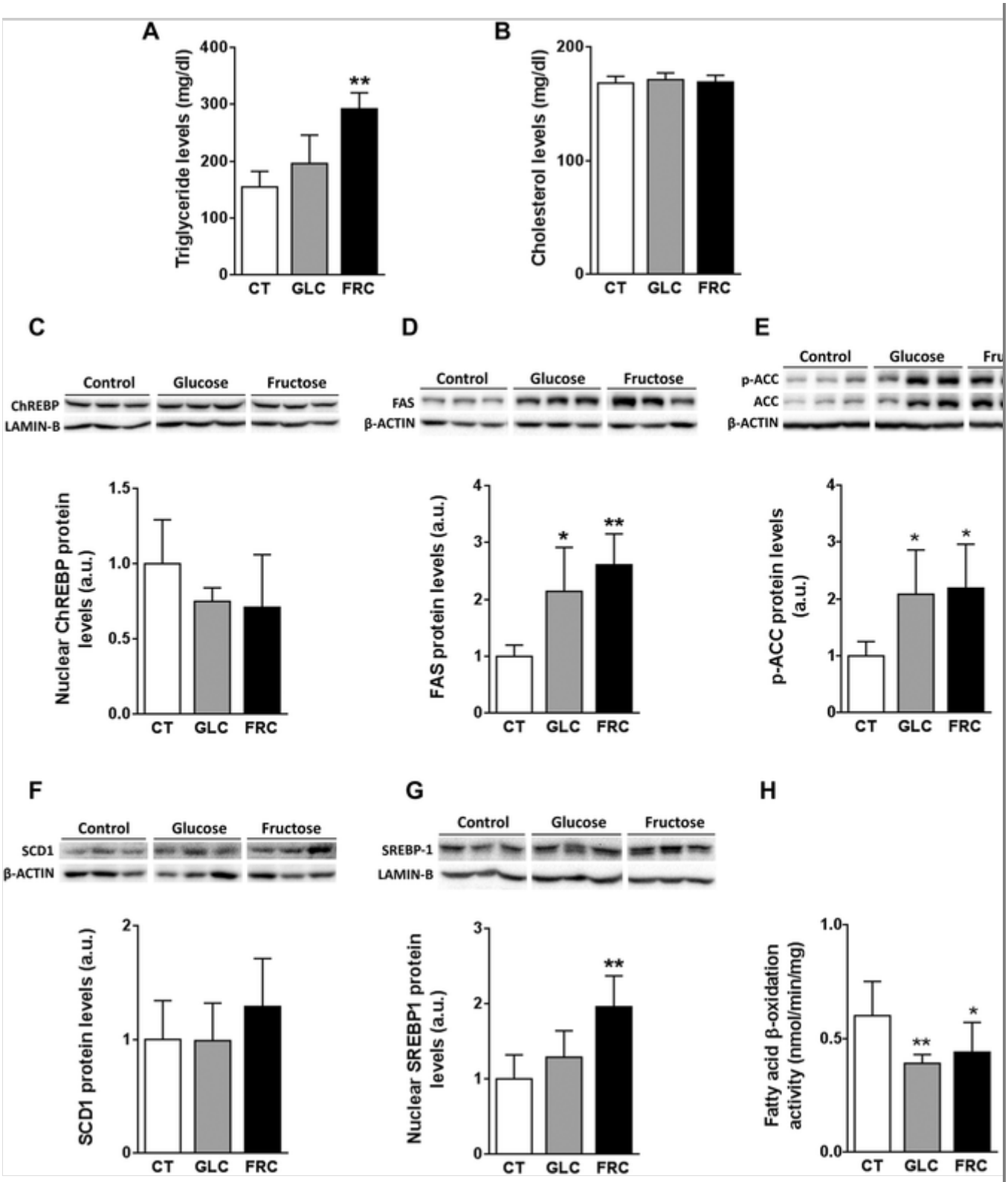
## Despite the lack of steatosis induction, only female rats consuming fructose showed marked hypertriglyceridemia after 7 months supplementation

After a prolonged 12-h fasting period, only fructose-supplemented rats showed a marked 1.9-fold increase in blood triglyceride concentrations vs control values, without modification of total cholesterol concentrations (Fig. 4a, b, respectively). Although the presence of carbohydrate response element binding protein (ChREBP) in nuclear extracts from liver samples was not modified (Fig. 4c), the expression of two key proteins involved in the de novo synthesis of fatty acids, i.e., fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), showed a marked increase in the livers of sugar-supplemented animals (Fig. 4d, e, respectively). As ChREBP is directly activated by intermediate sugar metabolites [30], the long fasting period probably accounted for the lack of increase in the ChREBP nuclear content; furthermore, a key lipogenic enzyme with a short half-life, such as stearoyl-CoA desaturase (SCD1) [31], which responds in a coordinated way to metabolic stimuli with FAS and ACC, also showed no increase in the livers of sugar-supplemented animals (Fig. 4f). The sustained hyperinsulinemia present in fructose-supplemented animals (see Supplemental Table 2) was probably responsible for the increased nuclear presence of sterol response element binding protein 1 (SREBP-1) in the livers of these animals (Fig. 4g), since the *srebpf1* gene is under the direct transcriptional control of insulin [32]. On the other hand, fatty acid β-oxidation activity decreased significantly in the livers of sugar-supplemented rats (Fig. 4h), probably due to the increased production of the allosteric inhibitor malonyl-CoA by ACC activation [33].

**Fig. 4**

Plasma triglyceride (**a**) and cholesterol (**b**) concentrations from CT (control female Sprague–Dawley—SD—rats), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group), and FRC (female SD rats supplemented with a 10% w/v fructose solution as

drinking water for 7 months) experimental groups; scatter plots represent individual values and the mean ± S.D for each group. Western-blot of nuclear ChREBP (**c**), FAS (**d**), phosphor- and total-ACC (**e**), SCD1 (**f**), and nuclear SREBP-1 (**g**) proteins, respectively, in liver samples obtained from CT, GLC, and FRC groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity of the phosphor- (ACC) or total-proteins. Representative bands shown in the upper part of the figure correspond to three different rats in each group. **h** Liver fatty acid β-oxidation activity in samples from CT, GLC, and FRC groups; scatter plots represent individual values and the mean ± SD for each group $*P < 0.05$, $**P < 0.01$ vs CT values



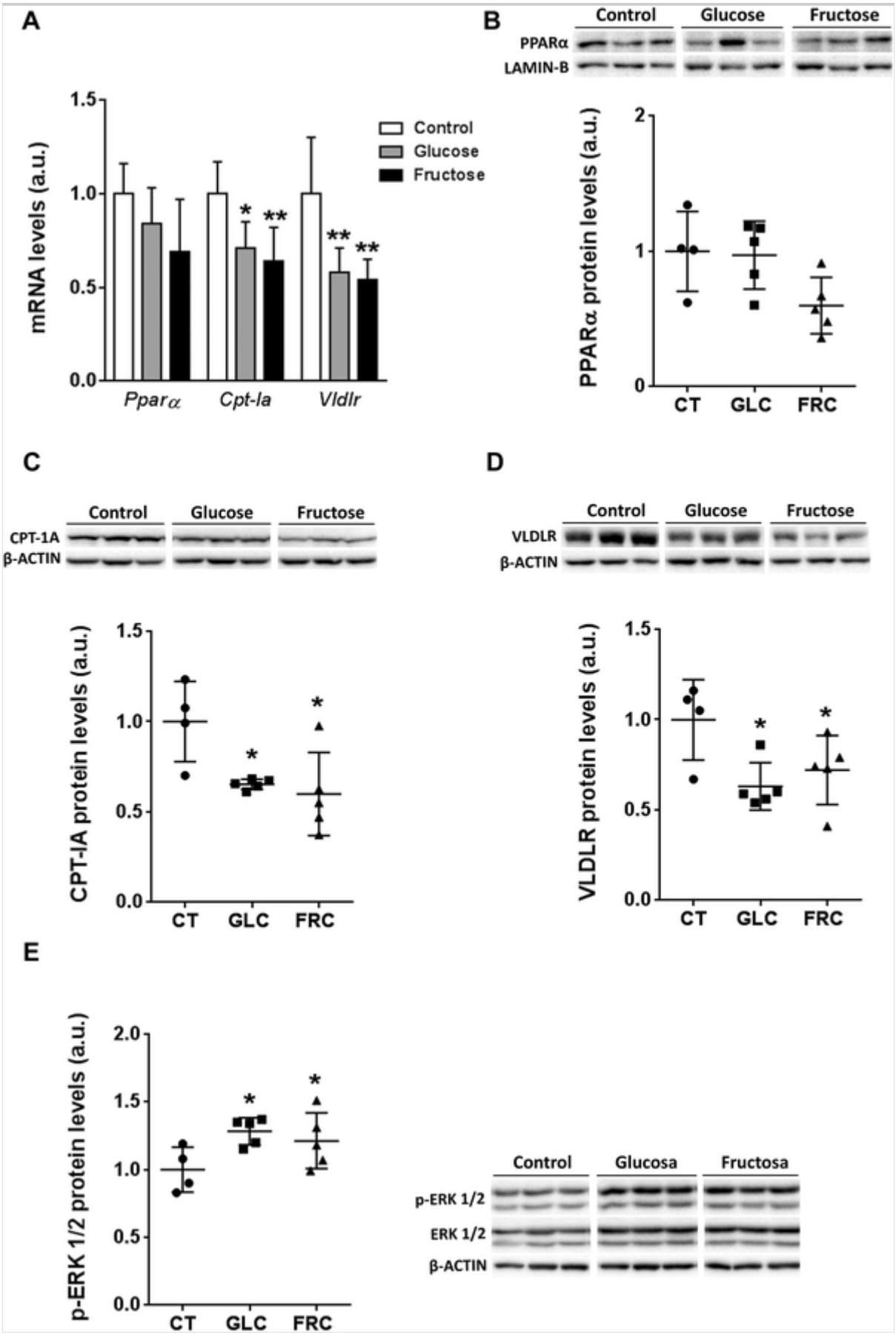

## Similarities and differential molecular signatures between livers from fructose- and glucose-supplemented female Sprague–Dawley rats

In the present study, after chronic supplementation (7 months), both sugars, glucose and fructose, did not significantly reduced liver PPARα expression and nuclear content (Fig. 5a, b), probably due to the dispersion of the individual data.

Nevertheless, both sugars reduced the liver expression of several genes that are targets of PPARα activity, such as *cptIa* (carnitine palmitoyl transferase-Ia, Fig. 5a, c) [34], *vldlr* (VLDL receptor—VLDLR—, Fig. 5a, d) [35], *pepck*, and *g6pc* (Fig. 3g–i) [36]. Qiu et al. [37] have described that in liver cells, high glucose/fructose concentration reduces PPARα activity and CPT-IA expression *via* aldose reductase expression and increased extracellular signal regulated kinase 1/2 (ERK1/2) activity; ERK1/2 phosphorylates and inhibits PPARα transcriptional activity. Indeed, in liver samples obtained from our chronically simple sugar-supplemented rats, there was a significant increase in total and phosphorylated and active (Fig. 5E) ERK1/2, thus given a possible explanation for reduced PPARα activity in these samples.

**Fig. 5**

**a** Bar plots representing the mean ± SD mRNA liver levels corresponding to the genes *pparα, cpt1a*, and *vldlr* from CT (control female Sprague–Dawley—SD—rats, $n = 6$), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group, $n = 8$), and FRC (female SD rats supplemented with a 10% w/v fructose solution as drinking water for 7 months, $n = 8$) experimental groups. Western-blot of nuclear PPARα (**b**), CPT-IA (**c**), VLDLR (**d**), and phosphor- and total-ERK1/2 (**e**) proteins, respectively, in liver samples obtained from CT, GLC, and FRC groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity of the phosphor- (ERK1/2) or total-proteins. Representative bands shown in the upper part of the figure correspond to three different rats in each group. $*P < 0.05$, $**P < 0.01$ vs CT values

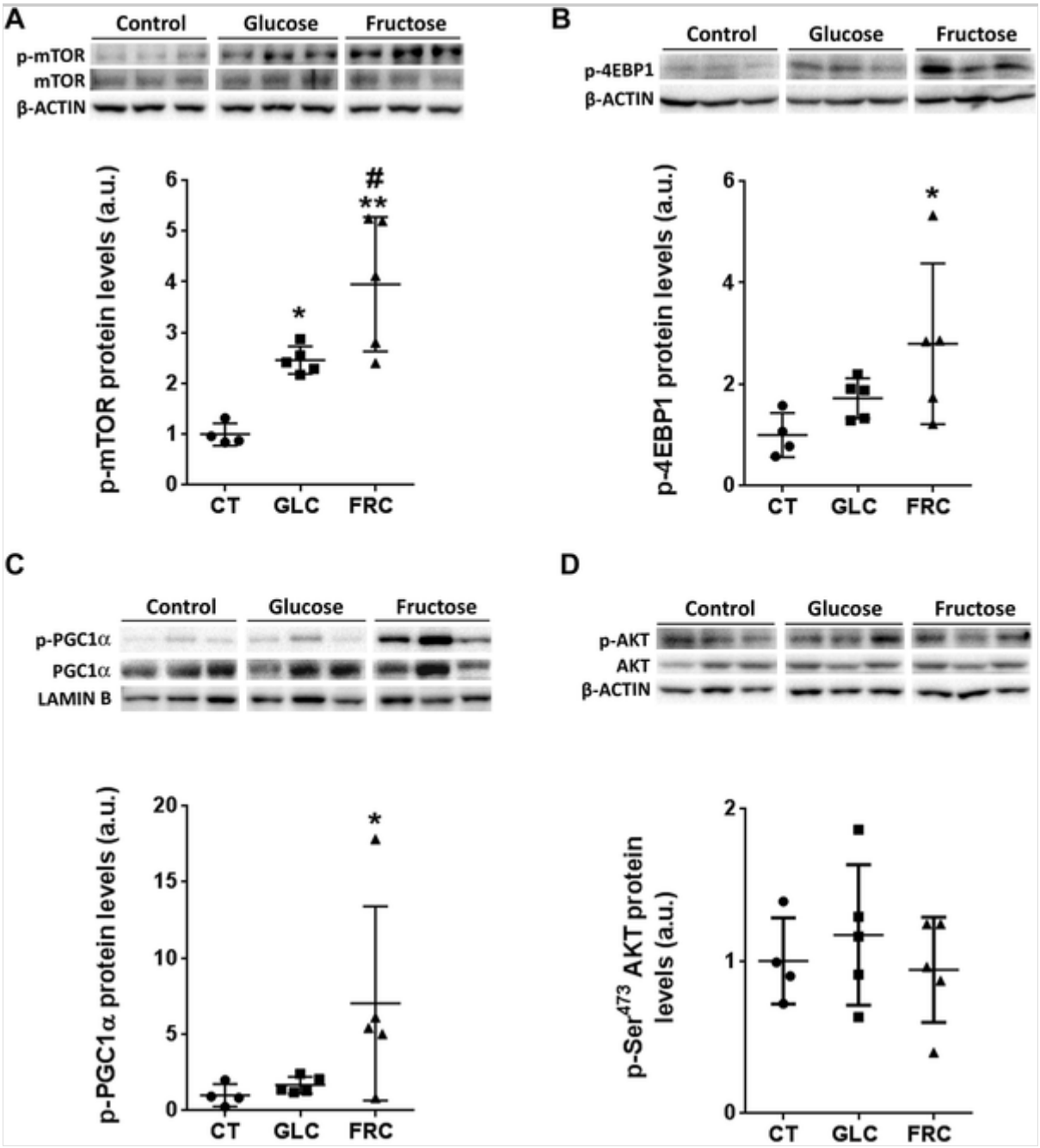



Furthermore, chronic supplementation with simple sugars significantly increased phosphorylation at serine 2481 of mammalian target of rapamycin (mTOR),

showing fructose a significant greater effect than glucose (Fig. 6a). Moreover, down path markers of mTOR complex 1 (mTORC1) activity [38], such as phosphor-eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1, Fig. 6b), and phosphor-peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, Fig. 6c), a direct target of mTORC1-driven ribosomal protein S6 kinase (S6K) activity [39], were consequently significantly increased in liver samples from fructose-supplemented animals. Mammalian target of rapamycin complex 2 (mTORC2) activity was not modified by simple sugar supplementation, as the degree of Akt phosphorylation at serine 473, a direct target of mTORC2 activity [38], was unmodified in these samples (Fig. 6d).

**Fig. 6**

Western blot of phosphor- and total-–mTOR (**a**), phosphor-4EBP1 (**b**), phosphor- and total-PGC1α (**c**), and phosphor ($Ser^{473}$)- and total-Akt (**d**) proteins, respectively, in liver samples obtained from CT (control female Sprague–Dawley —SD—rats), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group), and FRC (female SD rats supplemented with a 10% w/v fructose solution as drinking water for 7 months) experimental groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity of the phosphor proteins. Representative bands shown in the upper part of the figure correspond to three different rats in each group. *$P < 0.05$, **$P < 0.01$ vs CT values; $^{\#}P < 0.05$ vs GLC



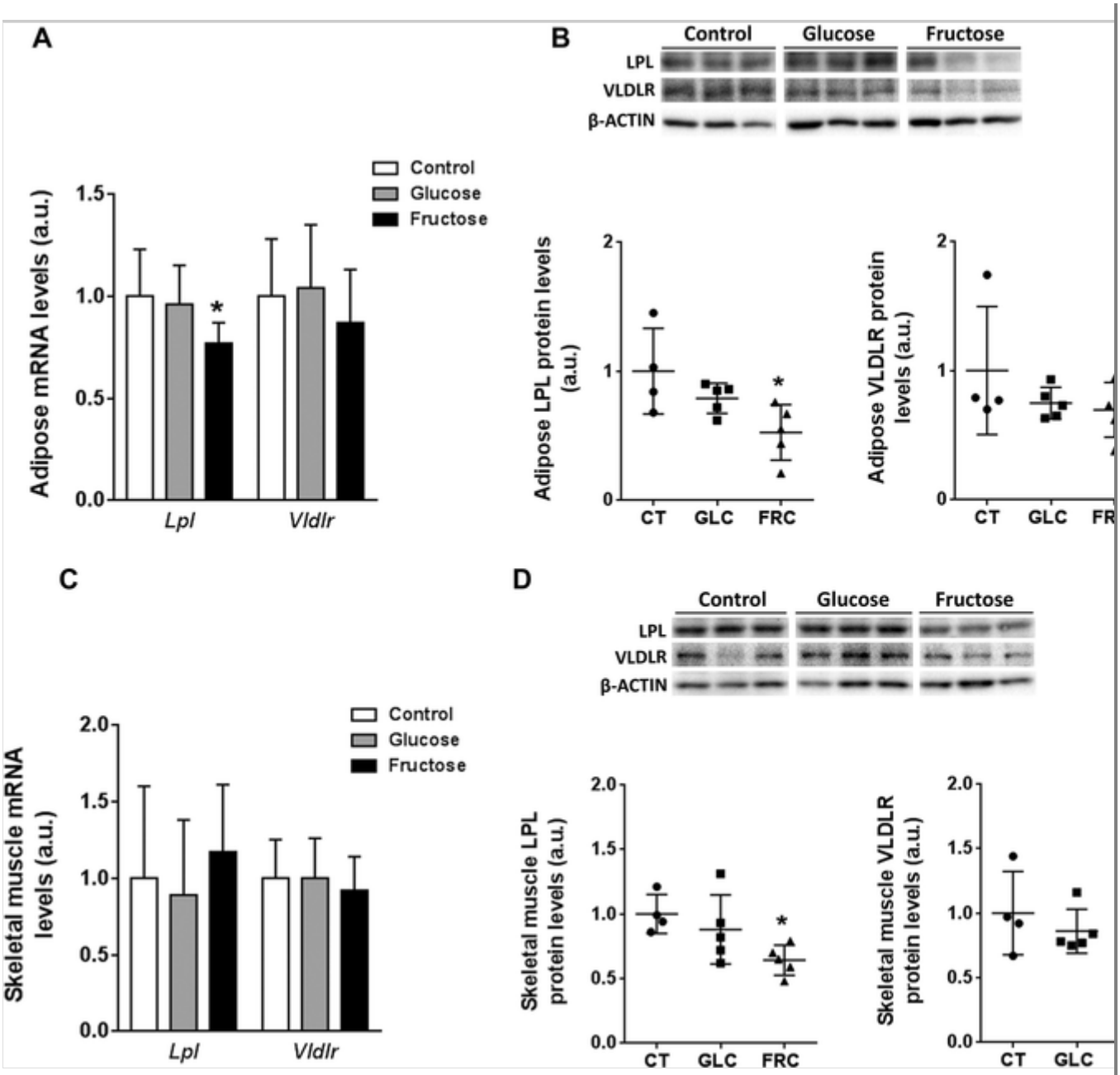

## Only fructose supplementation significantly diminished the expression of key proteins, such as VLDLR and lipoprotein lipase (LPL), involved in plasma-VLDL clearance

As shown previously, fructose and glucose supplementation similarly affected the liver expression of key lipogenic enzymes and the hepatic fatty acid beta oxidation activity. Despite that, only fructose-supplemented animals showed hypertriglyceridemia (see Fig. 4). Thus, we determined the expression of two key proteins, VLDLR and LPL, that control the clearance of plasma VLDL [35], in adipose tissue and skeletal muscle. As shown in Fig. 7, only fructose supplementation significantly diminished the tissue expression of both proteins, suggesting a reduction of VLDL clearance in these animals as determinant in the

manifestation of hypertriglyceridemia after chronic liquid fructose supplementation.

**Fig. 7**

**a** Bar plots representing the mean ± SD mRNA visceral adipose tissue levels corresponding to the genes *lpl*, and *vldlr* from CT (control female Sprague–Dawley —SD—rats, $n = 6$), GLC (female SD rats supplemented with a glucose solution prepared to match the number of calories ingested by the fructose group, $n = 8$), and FRC (female SD rats supplemented with a 10% w/v fructose solution as drinking water for 7 months, $n = 8$) experimental groups. **b** Western-blot of LPL, and VLDLR proteins, respectively, in visceral adipose tissue samples obtained from CT, GLC, and FRC groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity. Representative bands shown in the upper part of the figure correspond to three different rats in each group. **c** Bar plots representing the mean ± SD mRNA skeletal muscle levels corresponding to the genes *lpl*, and *vldlr* from CT ($n = 6$), GLC ($n = 8$), and FRC ($n = 8$) groups. **d** Western-blot of LPL, and VLDLR proteins, respectively, in skeletal muscle tissue samples obtained from CT, GLC, and FRC groups; scatter plots represent individual values of four–five different samples and the mean ± SD band intensity. Representative bands shown in the upper part of the figure correspond to three different rats in each group. *P<0.05 vs CT values.




# Discussion

In the present work, we demonstrate that chronic supplementation (7 months) with glucose or fructose in liquid form providing a clear excess of caloric intake in female rats does not result in steatohepatitis development and even evolved to the disappearance of fatty liver, a condition previously shown to develop in sub-chronic (2 months) supplementation with fructose. Furthermore, despite showing a reduction in the amount of IRS2, livers from sugar-supplemented rats had a marked suppression in the expression of gluconeogenic genes. Only fructose-, but not glucose-supplemented rats, clearly developed hypertriglyceridemia, probably related to a decreased clearance of triglyceride-rich lipoproteins.

Our data point to the selective increase in mTORC1 activity as a key molecular

signature of simple sugar supplementation. Although both sugar dietary supplementations provided exactly the same amount of calories, one of the main drivers of mTOR activation [40], fructose significantly increased the degree of mTOR phosphorylation to a greater extent than glucose. Two factors could be related to this situation: the hyperinsulinemia related to fructose supplementation, as insulin is a bona fide inductor of the mTORC1 pathway [40], and the hyperadiponectinemia related to glucose supplementation, as adiponectin, through AMPK activation [28], reduces mTORC1 activity [41]. Although we do not know the exact mechanism of glucose-induced hyperadiponectinemia in female Sprague–Dawley rats, this is a consistent effect that we have previously described [21].

Activation of mTORC1 can have deep consequences in liver glucose and fatty acid metabolism. Reduction of liver mTORC1 activity results in increased FoxO1 activation and enhanced hepatic gluconeogenesis [42, 43]; furthermore, it has been shown that, at least in mouse hypothalamus, mTORC1 directly controls FoxO1 phosphorylation and inactivation [44]. Thus, it could be assumed that the increased phosphorylation, and inactivation, of FoxO1 in livers of fructose-supplemented rats is directly related to the activation of the mTORC1 pathway and, as a consequence, the transcription of gluconeogenic genes, such as *g6pc* and *pepck*, will be reduced independently of the canonical insulin/IRS2/Akt/FoxO1 pathway [29]. Moreover, through S6K activity, the activation of mTORC1 results in the phosphorylation of PGC1α [39]. PGC1α is a transcriptional coactivator necessary for the expression of genes, such as *g6pc, pepck*, and *cptIα* controlled by transcription factors such as FoxO1 itself, hepatic nuclear factor 4 and PPARα [39, 45]. At least for the gluconeogenic genes, the phosphorylation of PGC1α through S6K attenuated its transcription-promoter activity [39].

Besides the possible interference of phosphorylated PGC1α in PPARα transcriptional activity, chronic simple sugar supplementation clearly increased liver ERK1/2 activity (see Fig. 5h), affording a plausive explanation of the reduced liver expression of PPARα-target genes, such as *cptIα* and *vldlr*, as it has been described that ERK1/2 phosphorylates and inhibits PPARα transcriptional activity [37]. Thus, chronic simple sugar supplementation, at least in the case of fructose, can reduce liver fatty acid oxidation by three complementary mechanism: decreased flux of substrates as a consequence of mTORC1-mediated inhibition of autophagy [8], malonyl-CoA-mediated allosteric inhibition of the rate-limiting fatty acid oxidative enzyme CPT-IA [33], and reduced expression of CPT-IA.

In the present chronic-supplementation study, besides both sugars, glucose and fructose, increased synthesis and decreased fatty acid oxidation, neither of them induced fatty liver and only fructose-supplemented animals showed a clear hypertriglyceridemia. The lack of hepatic steatosis after sugar supplementation could be the consequence of a healthy and selective activation of the UPR, mainly based in IRE1 and, in the case of fructose-supplemented animals, ATF6 activation, whereby misfolded or unassembled proteins are eliminated from the ER, without activation of an apoptotic response. ATF6 deletion and persistent CHOP expression, a downstream factor in the PERK branch of the UPR, correlates with unresolved stress and hepatic steatosis [46], while the heterodimerization of XBP1, a downstream factor in the UPR' IRE1 branch, and ATF6 as a transcription factor potentiates the induction of ER-associated degradation components, favoring ER homeostasis [25, 47]. Furthermore, it has been reported that an intense and specific response of the IRE1 branch prevents the appearance of hepatic steatosis, favoring the secretion of triglycerides incorporated in lipoproteins [48] and probably, when chronically sustained, the regression of preexisting steatosis. Anyhow, as a limitation of our study, it should be mentioned that Abdelmalek et al. [49] have reported that daily liquid fructose consumption, independently from age, sex, body mass index and total caloric intake, associates with lower steatosis grade and higher fibrosis stage in a sample of 427 adults enrolled in the NASH Clinical Research Network. As our study reflects a single time-frame vision (7 months) of a continuum process, we cannot discard that our fructose-supplemented rats could develop liver fibrosis with intakes prolonged in time.

VLDLR is a lipoprotein receptor responsible for the removal of Apo-E containing, triglyceride-enriched lipoproteins from plasma [50]. VLDLR deficiency reduces liver steatosis and promotes hypertriglyceridemia [51, 52]. At least in liver, the *vldlr* gene is under direct transcriptional control of PPARα [35]. Moreover, although PPARα does not seem to participate in the regulation of VLDLR expression in other tissues, such as adipose and skeletal muscle [35], VLDLR deficiency in these tissues relates to a deficit in LPL activity [52], further potentiating the deficit in plasma triglyceride-rich lipoproteins clearance. Thus, the fact that in the present study only fructose-supplemented rats showed a clear hypertriglyceridemia is most probably related to the combined fructose-related reduction of VLDLR and LPL expression in vWAT and skeletal muscle. Again, as it is known that adiponectin increases VLDLR and LPL in skeletal muscle [53], the mild hyperadiponectinemia induced after glucose supplementation could be responsible for preventing hypertriglyceridemia in these animals. Clearly, from

the results here shown, the differential effect of both sugars, fructose and glucose, on plasma adiponectin levels and its possible translation to humans deserves further studies.

In conclusion, the data here presented clearly demonstrate that in female rats, chronic liquid simple sugar supplementation in a background of a healthy solid rodent diet do not suffice to develop an estate of liver steatosis and its progression to NASH, as well as an increased expression of gluconeogenic enzymes, despite greatly increasing total caloric consumption. Besides, our study also clearly demonstrate that, at equivalent total calorie intake, fructose but not glucose, mainly due to its effects at extrahepatic tissues, is the only sugar capable of inducing and maintaining a clear hypertriglyceridemia, confirming that not only the amount of calories, but also the type of nutrient providing them, matters in the development of chronic metabolic diseases.

### Author contributions

GS and JCM were in charge of all experiments; MB contributed to liver triglyceride analysis and mRNA determination; NR contributed to Western blot experiments and prepared the figures; RMS helped in data interpretation and reviewed the manuscript; MA and JCL designed the experiments, analyzed the data, and wrote the manuscript.

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### Compliance with ethical standards

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

## Electronic supplementary material

Below is the link to the electronic supplementary material.

Supplementary material 1 (DOCX 20 KB)

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