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Ana Catarina Esteves Alves Costa Neves

Adult offspring's hepatic response to excess folic acid during perigestational period

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DESIGNAÇÃO DA ÁREA DO PROJECTO

Ciências exactas e naturais

TÍTULO DISSERTAÇÃO

Adult offspring's hepatic response to excess folic acid during perigestational period

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Faculdade de Medicina da Universidade do Porto, 10/03/2019

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Adult offspring's hepatic response to excess folic acid during perigestational period

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Abstract

A previous work from our group showed that perigestational high folic acid (FA) supplementation influences adult rat offspring to develop metabolic dysfunction and insulin-resistance. Our aim was to characterize the metabolic disruption in liver tissue of 13-month old offspring. The recommended dose of FA for pregnancy (C, 2 mg FA/kg of diet) or a high FA dose (HFA, 40 mg FA/kg of diet) were administered to Sprague–Dawley females during the perigestational period. At 10 months of age the progeny was divided into a group fed a standard diet and a group fed 10% fructose in the drinking water plus the standard diet until 13 months of age, when the animals were sacrificed. Liver pathological changes were evaluated by an experienced pathologist and hepatic steatosis was quantified by Oil-Red-O coloration, using ImageJ software®. The expression of hepatic genes involved in fatty acids synthesis, esterification and transport was analyzed by Real-time PCR. HFA exposure during perigestational period increased hepatic steatosis and tended to increase hepatic fibrosis, however it did not alter ballooning or inflammation in hepatic tissue. HFA treatment seemed to decrease the expression of fatty acids synthesis, esterification and metabolic transcription factors genes and tended to increase the expression of fatty acid transport protein 4. The results highlight the importance of the dose used for FA supplementation during pregnancy, as higher doses may induce hepatic steatosis and may indeed program the offspring for long term metabolic dysfunction.

Introduction

An emerging line of thought has driven attention to the importance of the intrauterine environment, not only because of the importance in early fetal development but also as a possible programmer of adulthood health and disease (Barua *et al.*, 2014b).

Folate is a water-soluble vitamin critical in DNA synthesis and DNA, RNA and protein methylation. Thus, folate is considered an important intervener in fetal programming (Barua *et al.*, 2014b).

In 1992, the United States Public Health Service recommended that all women in reproductive age should consume 0.4 mg of folic acid (FA) daily, to prevent neural tube defects (NTD) (1992). Currently, the World Health Organization (WHO) recommends women's supplementation with 0.4 mg/day of FA during

preconception, pregnancy and the three first months *post partum* and regulations for food mandatory fortification with FA are also in position in numerous countries (Crider *et al.*, 2011). Nowadays, several other products as bread, breakfast cereals and yeast spread are also FA fortified (Teixeira *et al.*, 2018) witch contributes to an even higher FA intake.

These public health interventions have raised concerns regarding potential adverse consequences of the excess of FA consumption that have been associated with changes in offspring development such as alterations in brain development (Barua *et al.*, 2014a), increased risk of autism (Wiens and DeSoto, 2017), infant asthma (Yang *et al.*, 2015), obesity (Huang *et al.*, 2014) and glucose intolerance (Huang *et al.*, 2014).

These concerns gain an even greater proportion in Portugal and other European countries where the FA dose given to women is approximately 12.5 times higher than the WHO recommended dose (Silva *et al.*, 2017).

Obesity prevalence together with metabolic syndrome (MetS) (characterized by insulin resistance, glucose intolerance, reduced high density lipoproteins levels, elevated triglycerides (TAG), and arterial hypertension) have been rising since the 1980s, imposing a significant health burden across the world (Inoue *et al.*, 2018).

In addition, non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease, affecting a quarter of the world's population (Ipsen *et al.*, 2018). NAFLD results from hepatic lipid accumulation caused by an imbalance between hepatic fat deposition and disposal, which is the end point of important pathways: uptake of circulating lipids from blood vessels, *de novo* lipogenesis (DNL), fatty acid oxidation, and lipids escapement (Ipsen *et al.*, 2018).

High fructose ingestion is a well-known factor contributing to adverse metabolic effects such as obesity (Johnson *et al.*, 2007) and NAFLD (Fisher *et al.*, 2017).

A previous work from our group showed that perigestational high folic acid (HFA) supplementation influences adult rat offspring to develop metabolic dysfunction and insulin-resistance. It was also observed an increased weight gain and an increase in food ingestion in HFA female offspring (Keating *et al.*, 2015).

Thus, our aim was to characterize the hepatic metabolic disruption in the adult offspring after perigestational supplementation with high levels of FA as well as to verify if the excess of FA increases susceptibility of offspring's metabolism to the presence of a metabolic challenge as the ingestion of excess of fructose. For this, we studied histopathological changes as well as lipid metabolic pathways in liver tissue of 13-month offspring, at which point the phenotypic alterations observed were the most exuberant.

The expression of several important genes of lipid metabolism and uptake was studied: acetyl-CoA carboxylase (*Acc*), the rate-limiting enzyme in fatty acid synthesis (Murray *et al.*); fatty acid synthase (*Fas)*; glycerol-3-phosphate acyltransferase 1 (*Gpat1*), which performs the first step in the esterification pathway (Murray *et al.*) and fatty acid transport proteins 3 and 4 (FATP3/4).

Finally, the expression of transcription factors critical in the regulation of lipid metabolic pathways, carbohydrate-responsive element-binding protein (*Chrebp*), sterol regulatory element-binding protein (*Srebp)-1c* and mammalian target of rapamycin (*mTor*), was assessed.

Methods

Animals treatment and diets

Animal treatment conditions were previously described in detail by our group (Keating *et al.*, 2015).

Briefly, Sprague–Dawley females were administered the recommended FA dose for pregnancy (C, 2 mg FA/kg of diet) (Achon *et al.*, 2000) or an high FA dose (HFA, 40 mg FA/kg of diet) which began at mating and stopped only at weaning, herein referred as the perigestational period. Diets of both groups were isocaloric, isolipidic, isoproteic, and isoglycidic. After the lactation period mothers and pups were given the same standard diet, containing 0.5 mg FA/kg of diet (the recommended daily intake dose).

At 10 months of age, progeny was divided into a group fed just a standard diet (C/STD and HFA/STD) and into another group where 10% fructose in the drinking water (C/FRU and HFA/FRU) was added until 13 months of age, when the animals were sacrificed for tissue collection. The organs were dissected, weighed and frozen in liquid nitrogen, and then kept at -80ºC until use, or fixed with 10% formaldehyde and prepared for anatomopathological analysis.

All animal procedures were approved by the Directorate of Health and Animal Protection Services of the General Veterinarian Direction of the Portuguese Government.

Histological staining and anatomopathological analysis

Liver tissue was fixed in buffered 10% formaldehyde, during 48h at 4˚C, dehydrated, embedded in paraffin and 4 µm thick sections were obtained with a Leica® Microtome (RM2125RT, Portugal).

To evaluate histological alterations one animal for each litter was randomly-selected and one liver section for each animal was stained with haematoxylin and eosin (H&E) (Sigma-Aldrich, Portugal).

Liver pathological changes were evaluated by an experienced pathologist in a blinded manner observing the entire tissue stained section.

Fibrosis, inflammation, steatosis, and ballooning were evaluated. The METAVIR scoring system was used to assess the extent of fibrosis with grade 0–4 (F0: no fibrosis; F1: portal fibrosis without septa; F2: portal fibrosis with few septa; F3: numerous septa without cirrhosis; F4: cirrhosis) Inflammation was evaluated in the centrilobular space, portal space and intralobular area and graded 0–3 (A0: no activity; A1: mild activity; A2: moderate activity; A3: severe activity). Steatosis was graded 0-3 (S0: less than 5% of the field; S1: between 5% and 33% of the field; S2: between 34% and 66% of the field; S3: more than 66% of the field) and ballooning graded 0-3 (B0: none; B1: few balloon cells; B2: many balloon cells; B3: exuberant ballooning) (Takahashi and Fukusato, 2014).

Fibrosis evaluation

Liver fibrosis was evaluated in tissue sections by Sirius Red histological staining in paraffin-embedded sections. The slides were incubated with a 0.5 % Sirius Red (Sigma-Aldrich, Portugal) solution dissolved in aqueous saturated picric acid for 90 minutes, washed in acidified water, dehydrated and mounted with Entellan.

Oil-Red-O staining

Hepatic lipid accumulation was quantified with Oil-Red-O (Sigma-Aldrich, Portugal) coloration in 12 µm liver cryo-sections, briefly counterstained with haematoxylin, using an optimized protocol (Mehlem *et al.*, 2013).

For each rat, ten randomly-stained sections were photographed (Nikon ECLIPSE 50i, UK). Lipid deposits were quantified by the number of red pixels in each camp captured and normalized to total area of the tissue section, using ImageJ software® (National Institute of Health, USA).

RNA extraction and quantification

Total RNA was extracted from liver samples, by grounding the tissue in liquid nitrogen and using NZYOL reagent (NZYTech, Portugal) followed by chloroform extraction and isopropanol precipitation. The quantity and quality of RNA generated was evaluated using a NanoDrop instrument (Thermo Scientific, Fisher Scientific, Portugal) by measurement of the optical density at 230 nm, 260 nm and 280 nm. All RNA samples were also tested for RNA degradation by electrophoresis on a denaturing agarose gel.

Gene expression analysis by quantitative real-time PCR

Total RNA was used for cDNA synthesis using NZYFirst-strand cDNA Synthesis kit (NZYTech, Portugal), according to the manufacturer's instructions, after RNA treatment (1 µg) with DNaseI (Promega RQ1 RNase-Free DNase, Promega, Spain), to avoid contamination with genomic DNA.

The hepatic genes expression was measured by Real-time PCR (qRT-PCR) using a LightCycler 96 (Roche, Portugal). Using 1 µL cDNA, 0.2 µL of each primer and 5 µL of SYBRGreen Master Mix (FastStart Essential DNA Green Master, Roche, Portugal) for which well of the 96-well plate, using the following conditions: One denaturation cycle (95˚C for 600 s), forty-five amplification cycles (95˚C for 10 s, annealing temperature (AT) for 10 s, and 72˚C for 10 s, with a single fluorescence measurement at the end) and one melting cycle (95˚C during 10 s, (AT+10) ˚C during 60 s, and 97 ˚C during 1 s with continuous fluorescence measurement) , in order to verify the quality of the transcription products.

The relative gene expression was calculated by the formula $2^{-(\overline{\text{Cq } gene\ of\ interest} - \overline{\text{Cq house keaping gene}})}$ (Schmittgen and Livak, 2008) using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as housekeeping gene.

Gene-specific primers (Sigma-Aldrich, Portugal) and conditions are listed in table 1.

Table 1 | Primers for Sprague–Dawley hepatic gene expression analysis by qRT-PCR.

Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Annealing Temperature (˚C)
Acc	*CCTTGTCAACGCATGGGCGG*	*GGCTTTGGGGTGGGGAGTCG*	*69*
Fas	*GCTGCAAGGAGCAAGGCGTG*	*CACACCGCCCGGCTGATACA*	*68*
Gpat1	*CACCACATCAAGGATACAGCTCA*	*AAGCCACAGTCAACCCAGTCC*	*62*
Chrebp	*TCATGGTGTCCTCGCCGCAC*	*TGGGAGACACCAGCTTGCCG*	*69*
Srebp1c	*GTGGGTCTCCTCCGAAGCCG*	*AGCATGTCTTCGATGTCGGTCAAG*	*66*
mTor	*GTACTGGCTACGTGGTGGAG*	*TAACACTCGGATGGCCTCTC*	*58*
Gapdh	*GGCATCGTGGAAGGGCTCA*	*ATGCCAGTGAGCTTCCCGT*	*70*
Fatp3	*TTCTGAGAACTTGCCACCGT*	*AAGCCCTCATTTGCCATCCT*	*71*
Fatp4	*TGCACAGCAGGTATTACCGA*	*TGGCCAATCCCCACAATGTT*	*72*

Acc – acetyl-coA carboxylase; *Fas* – fatty acid synthase; *Gpat1* - glycerol-3-phosphate acyltransferase 1; *Chrebp* - carbohydrate-responsive element-binding protein; *Srebp1c* - sterol regulatory element-binding protein 1c; *mTor*- mammalian Target of rapamycin; *Gapdh* - glyceraldehyde-3-phosphate dehydrogenase; *Fatp3*- fatty acid transport protein 3; *Fatp4*- fatty acid transport protein 4.

Calculations and statistics

Data are expressed as arithmetic mean ± standard error of the mean (SEM). For comparisons between two groups, Student's t-test was used. Differences between various treatment groups were evaluated by the two-way ANOVA, followed by Tukey's test, enabling the assessment of statistical significance regarding HFA effect, metabolic challenge (high fructose feeding), and the interaction between these two factors. Differences were considered significant, at a confidence interval of 95%, whenever $p<0.05$. All statistical analysis was performed using GraphPad Prism 7 statistical software (GraphPad Software Inc., USA). The sample size (n) corresponds to the number of different litters analyzed.

Results

HFA treatment increased hepatic steatosis in adult offspring

Hepatocellular steatosis is the hallmark of NAFLD and reflects lipid accumulation in liver. So, steatosis was evaluated and graded 0-3 in a liver section stained with H&E.

HFA exposure during perigestational period induced hepatic steatosis in adult offspring when compared with adult offspring which mothers were exposure to the FA recommended dose during perigestational period (control groups) (p=0,0166, two-way ANOVA) (Figure 1).

Fructose is a well-known inductor of metabolic dysfunction, inducing dyslipidemia and insulin resistance (Stanhope *et al.*, 2009). Fructose exposure showed a tendency to increase hepatic steatosis when compared with the standard diet, despite the differences were not statistically significant (Figure 1). In addition, the HFA background did not significantly alter the effect of the metabolic challenge on the steatotic degeneration.

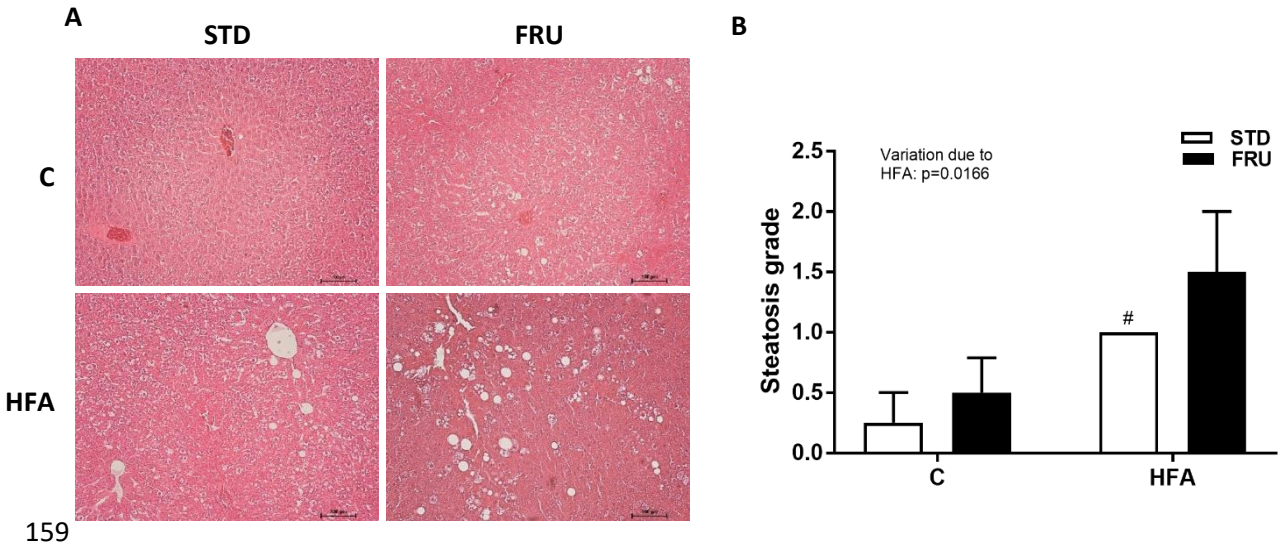


Figure 1 – Evaluation of hepatic steatosis by H&E stained liver section of 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. **(A) Representative images of hepatic lipid accumulation for each group of treatment**. Scale bar: 100 µm. Magnification 10x. **(B) Hepatic steatosis quantification**. Liver pathological changes were evaluated by an experienced pathologist in a blinded manner that observed the entire tissue stained section and steatosis was graded 0 to 3. Statistical analysis included two-way ANOVA followed by the Tukey's test and Student's t-test (# $p<0.05$ vs C/STD); n=4.

Steatosis was also quantified with Oil-Red-O staining, using ImageJ software®, and the percentage of area corresponding to lipid deposits was obtained for each section.

Oil-Red-O staining corroborated H&E results: both HFA exposure and fructose diet increased hepatic steatosis (p=0,0133 and p=0,0320, two-way ANOVA, respectively).

Again, the HFA background did not change hepatocellular steatosis in response to fructose challenge (Figure 2).

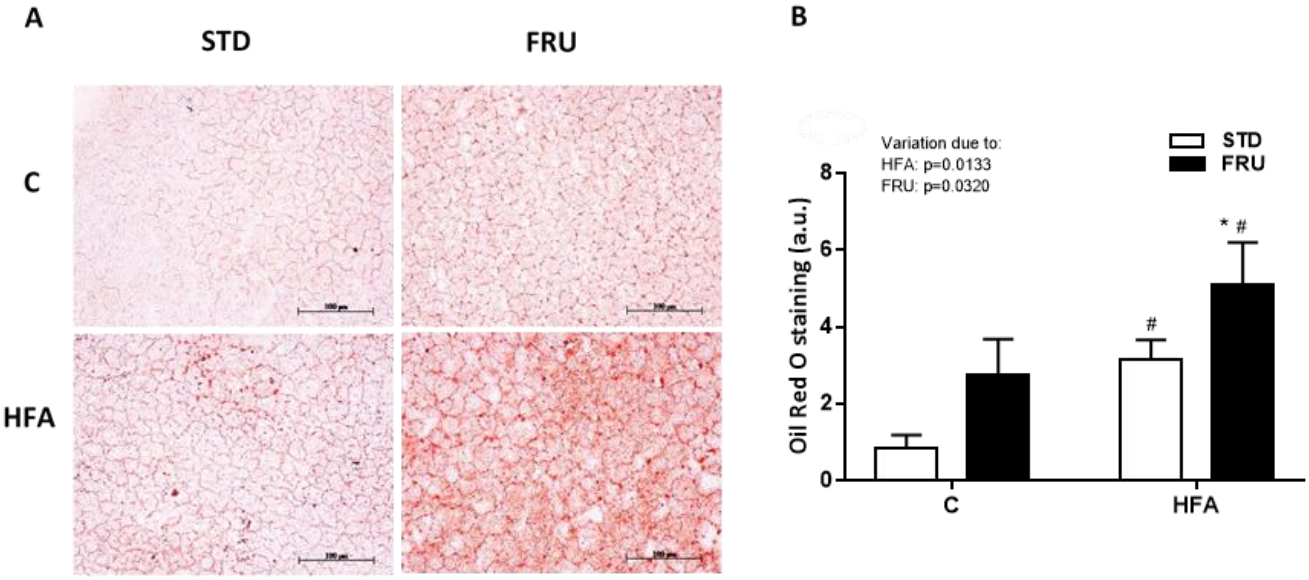


Figure 2 -Hepatic lipid accumulation evaluated by Oil-Red-O staining of 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. **(A) Representative images of hepatic lipid accumulation for each group of treatment.** Scale bar: 100 µm. Magnification 20x. **(B) Quantification of Oil-Red-O staining**, using ImageJ software®, and the area percentage corresponding to lipid deposits was obtained for each section. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test (* $p<0.05$ vs C/STD) and t student test (# $p<0.05$ vs C/STD); $4 \leq n \leq 5$; a.u., arbitrary units.

HFA treatment does not alter ballooning degeneration

Ballooning degeneration refers to liver parenchymal cell death and is generally considered a form of hepatocyte apoptosis associated to a context of fatty liver inflammation.

Neither HFA nor fructose exposure changed the ballooning degeneration of hepatocyte when compared with C/STD group (Figure 3). The HFA background also did not change tissue ballooning in response to the fructose challenge.

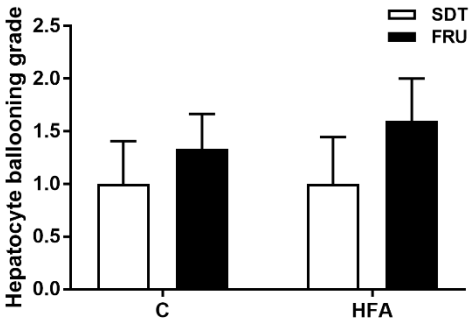
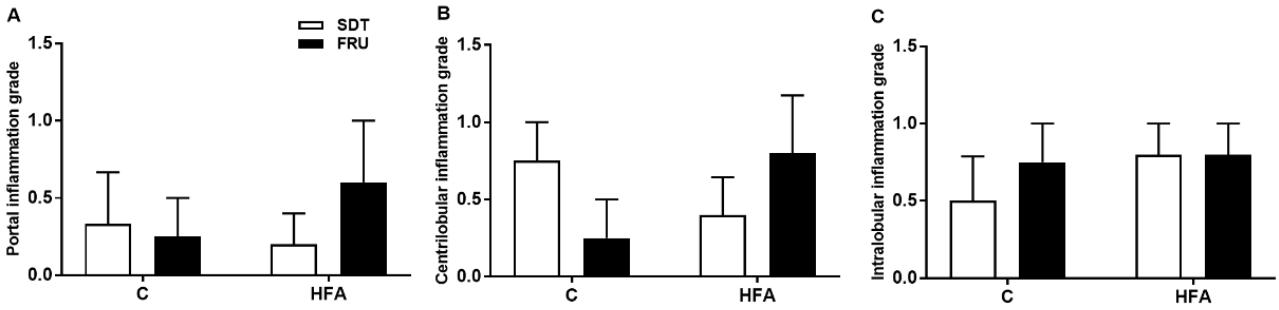


Figure 3- **Hepatocyte ballooning** in 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. Liver pathological changes were evaluated by an experienced pathologist in a blinded manner observing the entire tissue stained section and ballooning was graded 0 to 3. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test and t student test; $3 \leq n \leq 5$.

HFA treatment does not change inflammatory activity in hepatic tissue

Hepatic steatosis in a metabolic dysfunction context is often associated to an increase in inflammatory activity. So, inflammation of either portal, intralobular or centrilobular compartment was evaluated.

HFA exposure did not change the inflammatory activity of either portal, intralobular or centrilobular compartment when compared with the C/STD group. Fructose feeding tends to increase portal and centrilobular inflammation in animals with a background of perigestational HFA but not in control animals. Nevertheless, the interaction of HFA and fructose effects is not statistically significant (Figure 4).



202 Figure 4- Hepatic inflammation in 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for
203 pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a
204 group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. **(A)**
205 **Portal inflammation; (B) Centrilobular inflammation; (C) Intralobular inflammation.** Liver pathological changes were evaluated by an
206 experienced pathologist in a blinded manner observing the entire tissue stained section and inflammation was graded 0 to 3 in each
207 compartment. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test and t
208 student test; 3≤n≤5.
209

210 ## HFA treatment seemed to increase hepatic fibrosis

211 Liver fibrosis is the excess of extracellular matrix proteins accumulation, including collagen, that
212 results from chronic inflammatory reactions in the liver (Wynn, 2008). Its evaluation was performed using
213 metavir score, one of the most widely accepted scoring systems for assessment of fibrosis and
214 necroinflammation in chronic liver disease (Shiha and Zalata, 2011).

215 Metavir score tended to be higher in HFA group (0,80 ± 0,37) when compared with control group
216 (0,25 ± 0,25) (figure 5D), although the difference was not statistically significant.

217 This tendency may be the reflection of an increase of portal and centrilobular fibrosis that seemed to
218 have the same tendency and profile, although not statistically significant (Figures 5A and 5B). Periportal
219 fibrosis revealed no changes with treatments.

220 As it can be seen in figure 5, fructose treatment also seemed to increase hepatic fibrosis, although
221 the results did not reach statistical significance.

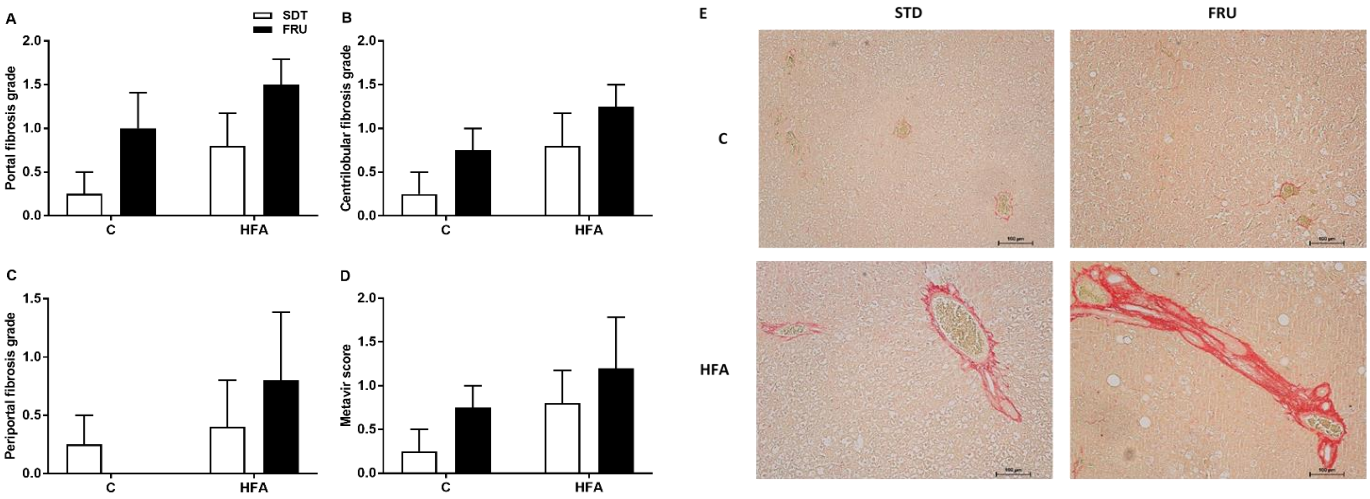


Figure 5- Hepatic fibrosis evaluated by Sirius Red stained liver in 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. **(A) Portal fibrosis quantification; (B) Centrilobular fibrosis quantification; (C) Periportal fibrosis quantification; (D) Metavir score quantification; (E) Representative images of hepatic fibrosis for each group of treatment.** Scale bar: 100 µm. Magnification 10x. Liver pathological changes were evaluated by an experienced pathologist in a blinded manner observing the entire tissue stained section and fibrosis was graded 0 to 4 in each compartment. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test and t student test; 4≤n≤5.

HFA treatment decreased the expression of fatty acid synthesis genes.

The alterations described for liver tissue could be a result of lipid dysmetabolism of the offspring. So, hepatic gene expression of key enzymes and transcript factors involved in lipid metabolism were evaluated by qRT-PCR.

HFA exposure decreased *Acc* gene expression when compared with the control group (Figure 6A) (p=0,0132, two-way ANOVA). On the contrary, fructose exposure did not modify *Acc* gene expression when compared with the standard diet treated animals, even under HFA background.

Fas gene expression also tended to decrease with the exposure to HFA, as well as with fructose ingestion, despite this effect did not reach statistical significance (Figure 6B). It seems that HFA background

tended to attenuate the metabolic challenge effect on *Fas* expression, although interaction did not reach statistical significance.

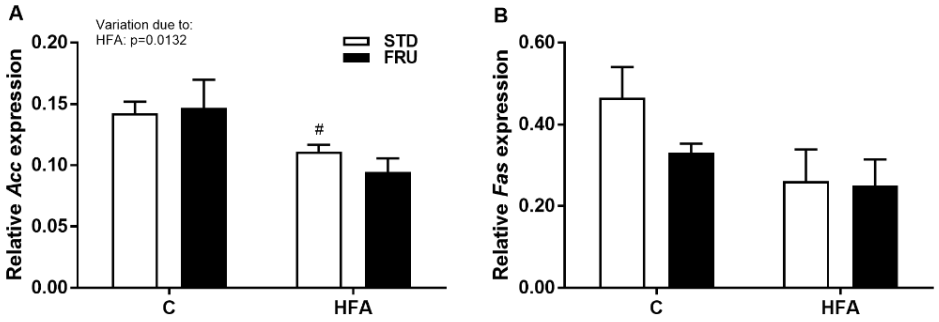


Figure 6- Fatty acid synthesis genes expression in hepatic tissue of 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. **(A) Acetyl-coA carboxylase (*Acc*); (B) Fatty acid synthase (*Fas*).** Genes expression was assessed by real time PCR and normalized to *Gapdh*. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test and t student test (# $p<0.05$ vs C/STD); $3 \leq n \leq 5$.

HFA treatment tended to decrease the expression of fatty acid esterification genes.

The esterification of fatty acids transported to liver or produced there is promoted by *Gpat1*.

As can be seen in figure 7, both HFA exposure and fructose challenge appear to decrease *Gpat1* expression, although the differences did not reach statistical significance. The HFA background appeared to attenuate the metabolic challenge effect on the *Gpat1* expression.

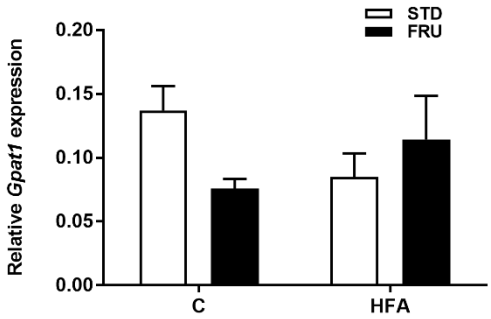


Figure 7- **Glycerol-3-phosphate acyltransferase 1 (*Gpat1*)** gene expression in hepatic tissue of the 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. *Gpat1* expression was assessed by real time PCR and normalized to *Gapdh*. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test and t student test; 3≤n≤5.

HFA treatment decreases the expression of metabolic transcription factors.

Both *Srebp1c* and *Chrebp* play synergic roles activating lipogenic pathways (Eberle *et al.*, 2004, Iizuka, 2017).

The expression of *Srebp1c* and *Chrebp* demonstrated a tendency to decrease with the exposure to HFA and also to fructose, despite this difference was not statistically significant (Figure 8A and 8B). Curiously, the response the ingestion of a fructose-rich diet seemed to be different in presence or absence of an HFA background. HFA background tended to decrease the effect of metabolic challenge caused by high fructose ingestion for both *Srebp1c* and *Chrebp* expression, although interaction was not statistically significant.

mTor has been implicated in the development of metabolic diseases because it modulates several metabolic pathways, including those regulated by *Srebp1c* and *Chrebp*.

Corroborating the above described metabolic gene expression results, HFA exposure induced a statistically significant downregulation of *mTor* expression (p=0,0050, two-way ANOVA; p=0,0088, Tukey's test for HFA/STD group vs C/STD group) (Figure 8C). Fructose ingestion by control animals was also able to significantly reduce *mTor* expression (p<0,0001, two-way ANOVA; p=0,0003, Tukey's test for C/FRU group versus C/STD).

An interaction between HFA and fructose-rich diet effects was observed regarding this gene expression, as the perigestational HFA background attenuated the downregulation of *mTor* expression in the presence of the metabolic challenge (p=0,0217, two-way ANOVA).

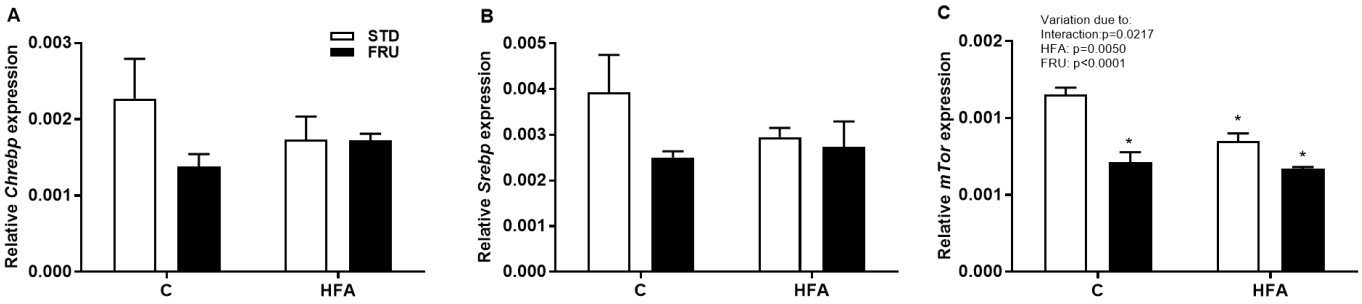


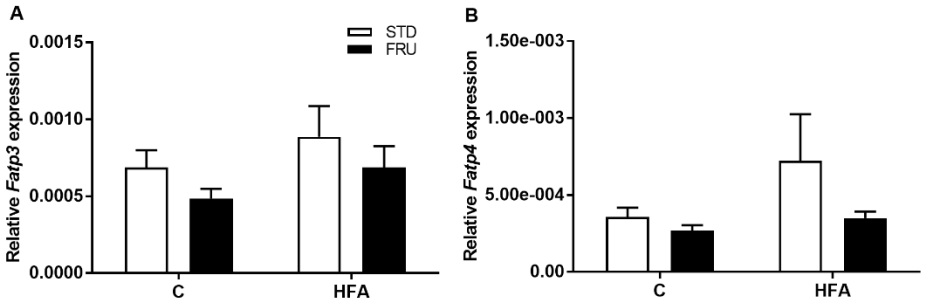
Figure 8- Expression of transcription factors genes in hepatic tissue of the 13-month Sprague-Dawley which mothers were exposure to the recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet (FRU) until 13 months of age. **(A) Carbohydrate-responsive element-binding protein (*Chrebp*); (B) Sterol regulatory element-binding protein (*Srebp1c*); (C) mammalian target of rapamycin (*mTor*).** Genes expression was assessed by real time PCR and normalized to *Gapdh*. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA followed by the Tukey's test (* p<0,005 vs C/STD); 3≤n≤5.

HFA treatment tended to increase the expression of fatty acid transport protein

Liver uptake of circulating lipids, as well as DNL, is involved in NASH pathogenesis. *Fatp3 and Fatp4* are expressed in endothelial cells but also in hepatocytes and are involved in fatty acids uptake by the liver (Glatz *et al.*, 2010, Mehrotra *et al.*, 2014).

Fatp3 gene expression was not affected by HFA or fructose exposure (Figure 9A).

Interestingly, *Fatp4* gene expression seemed to be higher in HFA group (0,0007234± 0,0003019) when compared with control group (0,0003563± 0,00006204) as can be observed in figure 9B, although the difference was not statistically significant. Fructose exposure did not modify the *Fatp4* gene expression when compared with the standard diet treated animals, even under HFA background.



298 Figure 9- Expression of transcription factors genes in hepatic tissue of 13-month Sprague-Dawley which mothers were exposure to the
299 recommended folic acid dose for pregnancy (C) or to a high folic acid dose (HFA) during the perigestational period. Offspring were
300 divided at 10 months of age into a group fed standard diet (STD) and a group fed 10% fructose in the drinking water plus standard diet
301 (FRU) until 13 months of age. **(A) Fatty acid transport protein 3 (*Fatp3*); (B) Fatty acid transport protein 4 (*Fatp4*).** Genes expression was
302 assessed by real time PCR and normalized to *Gapdh*. Values are presented as mean ± SEM. Statistical analysis included two-way ANOVA
303 followed by the Tukey's test and t student test; 3≤n≤5.
304

305 ## Discussion

306 A previous work from our group showed that perigestational high folic acid (HFA) supplementation
307 influences adult rat offspring to develop metabolic dysfunction and insulin resistance (Keating *et al.*, 2015).
308 Insulin resistance is associated to ectopic lipid deposition causing hepatic steatosis and NAFLD, impaired
309 insulin secretion and cardiomyopathy and coronary heart disease (Saponaro *et al.*, 2015).

310 So, this study aimed to characterize the metabolic disruption programed by HFA perigestational
311 exposure in adult offspring, analyzing the phenotypic alterations that occurred in the liver of these animals
312 as well as the underlying molecular pathways. Table 2 summarizes the main results of this work.

Table 2 | Effect of HFA and FRU exposure on Sprague–Dawley hepatic histology and gene expression.

	Folic Acid Effect		Fructose Effect		Interaction	
Hepatic steatosis	↑	p=0,0166	↑	p=0,2563	↔	p=0,6981
Ballooning degeneration	↔	p=0,7625	↔	p=0,2996	↔	p=0,7625
Portal inflammation	↔	p=0,7404	↔	p=0,6291	↔	p=0,4637
Centrilobular inflammation	↔	p=0,7416	↔	p=0,8689	↔	p=0,1524
Intralobular inflammation	↔	p=0,4632	↔	p=0,5985	↔	p=0,5985
Portal fibrosis	↑	p=0,1530	↑	p=0,0562	↔	p=0,9435
Centrilobular fibrosis	↑	p=0,1076	↑	p=0,1419	↔	p=0,9357
Periportal fibrosis	↔	p=0,2723	↔	p=0,8594	↔	p=0,4473
Metavir score	↑	p=0,2562	↑	p=0,3047	↔	p=0,9074
***Acc* expression**	↓	p=0,0132	↔	p=0,6621	↔	p=0,4722
***Fas* expression**	↓	p=0,0653	↔	p=0,3204	↓	p=0,3958
***Gpat1* expression**	↓	p=0,8198	↓	p=0,5947	↓	p=0,1483
***Chrebp* expression**	↓	p=0,7404	↓	p=0,1319	↓	p=0,1413
***Srebp* expression**	↓	p=0,5188	↓	p=0,1727	↓	p=0,3032
***mTor* expression**	↓	p=0,0050	↓	p<0,0001	↓	p=0,0217
***Fatp3* expression**	↔	p=0,2269	↔	p=0,2251	↔	p=0,9951
***Fatp4* expression**	↑	p=0,2138	↔	p=0,2014	↔	p=0,4158

Acc – acetyl-coA carboxylase; *Fas* – fatty acid synthase; *Gpat1* - glycerol-3-phosphate acyltransferase 1; *Chrebp* - carbohydrate-responsive element-binding protein; *Srebp1c* - sterol regulatory element-binding protein 1c; *mTor*- mammalian Target of rapamycin; *Fatp3*- fatty acid transport protein 3; *Fatp4*- fatty acid transport protein 4; ↑, increase; ↓, decrease; ↔, no change detected;

Our most striking finding was that HFA exposure of mothers during the perigestational period programmed for increased hepatocellular steatosis, the central pathological lesion of NAFLD, in 13-month-old offspring. This reflects, hepatic lipid unbalance favoring ectopic lipid accumulation, an alteration that has been associated with insulin resistance (Abdul-Wahed *et al.*, 2017), also observed in these animals (Keating *et al.*, 2015).

Hepatocellular steatosis can be macrovesicular, characterized by large fat droplet filling the hepatocyte and displacing the nucleus (Tandra *et al.*, 2011) and usually present in NAFLD (Takahashi and Fukusato, 2014), or microvesicular, characterized by small intracytoplasmic liposomes (Tandra *et al.*, 2011)

328 and usually correlated with a more advanced histologic stage of NAFLD (Tandra *et al.*, 2011). Both
329 macrovesicular and microvesicular steatosis were observed in this study (data not shown).

330 Despite there are no similar studies focusing on the effect of perigestational FA supplementation in
331 offspring's liver, HFA has been shown to affect hepatic lipid handling in other study set-ups. For example,
332 Burdge, et. al have shown that HFA supplementation of rats during puberty increases hepatic TAG
333 concentration compared with offspring fed a diet containing the adequate folate irrespective of maternal
334 diet (Burdge *et al.*, 2009). More recently, Christensen KE, et.al have suggested that HFA consumption in adult
335 mice leads to altered lipid metabolism and liver injury as well as increased liver weight. Additionally, the
336 authors show the appearance of anomaly large degenerating cells in liver, consistent with NAFLD
337 (Christensen *et al.*, 2015).

338 NAFLD can be classified histologically into (a) nonalcoholic fatty liver (NAFL) - characterized by
339 hepatocellular steatosis without marks of cellular injury, represented by hepatocyte ballooning- and (b)
340 nonalcoholic steatohepatitis (NASH) - characterized by the presence of inflammatory activity and steatosis
341 with hepatocyte ballooning with or without fibrosis (Chalasani *et al.*, 2018).

342 In this study, HFA exposure during the perigestational period also tended to increase hepatic fibrosis,
343 an irreversible mark of liver injury compatible with the progressive nature of the disease. Fibrosis in NAFLD
344 is frequently accompanied by an active necroinflammatory reaction (hepatic inflammation and hepatocyte
345 ballooning). However, in this case we observed fibrosis without active lesions which may also occur,
346 suggesting previous episodes of steatohepatitis (Takahashi and Fukusato, 2014).

347 The characteristic fibrosis pattern in NASH occurs in pericellular area, but as NAFLD progresses portal
348 fibrosis installs, which was in fact the most common pattern observed in livers of the offspring in our study,
349 suggesting a later stage of liver injury in our animals. The most important histological factor for prognosis of
350 NAFLD is fibrosis, specifically, bridging fibrosis (stage 3) or cirrhosis (stage 4), both associated with higher
351 mortality (Chalasani *et al.*, 2018). Bridging fibrosis, characterized by
352 bands of fibrous tissue and collagen, seen by low-power light microscopy, which connect portal spaces
353 and centrilobular spaces, was present in some of our HFA animals, corroborating the idea of disease
354 progression (Takahashi and Fukusato, 2014).

As hepatic lipid accumulation may result from an imbalance of several pathways, such as uptake of circulating lipids from blood vessels, DNL, fatty acid oxidation, and export of lipids (Ipsen *et al.*, 2018), the expression of several key genes of these pathways was studied.

mTor is a protein kinase which regulates hepatic lipid metabolism, being involved in the response to environmental features, and it has been implicated in several pathological processes such as cancer, obesity, and T2DM (Laplante and Sabatini, 2012). *mTor* has also been pointed out as a folate sensor in placenta, regulating nutrient transport and metabolism, functioning as a link between maternal folate status and fetal growth (Rosario *et al.*, 2017).

Perigestational HFA exposure decreased hepatic *mTor* expression in the offspring. This result is in strong agreement with the observed trend for *Srebp* and *Chrebp* downregulation as well as with the downregulation of their downstream lipogenic genes: *Acc*, *Fas* and *Gpat1*. This corroborates *mTor, Srebp* and *Chrebp* as master regulators of lipogenic enzymes in the liver.

Corroborating this idea, *mTor* inhibition has been reported in liver macrophages of NASH patients (Liu *et al.*, 2019) and also in smooth muscle vascular cells following FA supplementation (Pan *et al.*, 2017).

Curiously, it has been reported that polyunsaturated fatty acids may decrease SREBP by stimulating *Srebp1c* mRNA degradation in the liver (Duarte *et al.*, 2014). Also, although high *Chrebp* expression appeared in NASH patient's biopsies, *Chrebp* levels decreased in patients with high insulin resistance (Ipsen *et al.*, 2018).

Nevertheless, these results are apparently contradictory with the observed hepatic steatosis.

Indeed, some reports have demonstrated an increase in hepatic lipid accumulation with concomitant sustained *mTor* activation (Kubrusly *et al.*, 2010), probably in response to high circulating levels of nutrients and pro-inflammatory cytokines (Laplante and Sabatini, 2012). In addition, some recent studies have been linking the increase in *mTor* expression to hepatocarcinogenesis associated with metabolic syndrome and NASH (Umemura *et al.*, 2014, Okuno *et al.*, 2018).

In this context, we hypothesize that the observed decrease in *mTor, Srebp* and *Chrebp* resulting in the downregulation of the lipogenic pathway may reflect a compensatory effect for the observed hepatic lipid

accumulation which seems to result from increased fatty acid uptake, as suggested by the trend in *Fatp4* upregulation by perigestational HFA.

Interestingly, some human studies reveal that DNL contribute only by 25% to total liver lipids in patients with NAFLD (Abdul-Wahed *et al.*, 2017). So, the study of other pathways that could be involved in the pathophysiology of NAFLD such as circulating fatty acids and its uptake by the liver, which account for approximately 60% of the intrahepatic TAG in NASH (Ter Horst and Serlie, 2017), is critical in this context.

This increased hepatic lipid accumulation could additionally result from a putative increase in lipid retention, possibly due to alterations in liver β-oxidation, very low-density lipoprotein (VLDL) secretion that have also been implicated in obesity and insulin resistance pathogenesis (Duarte *et al.*, 2014). The impact of perigestational HFA upon these later pathways should be further explored.

Importantly, the inhibition of *Chrebp,* by RNAi or gene delection, has been shown to revert hepatic steatosis in obese and insulin-resistant mice (Abdul-Wahed *et al.*, 2017), suggesting that the decreased expression of *Chrebp* observed in liver's offspring of HFA treated mothers may indeed represent a compensatory mechanism protecting the liver from further damage.

Also critical in the context of our proposed compensation mechanism is that GPAT1 deficiency has been proposed to protect against diet-induced hyperlipidemia and hepatic steatosis (Takeuchi and Reue, 2009).

So, although we observed an increase in hepatic steatosis in HFA/STD animals, all the obtained results regarding metabolic pathways suggest that DNL pathways were downregulated at the final of the experiment. These alterations may indeed represent a compensatory mechanism against hepatic lipid accumulation in a less active, later phase of the disease. In the particular case of our study set-up, it seems that HFA offspring had a more active/ inflammatory phase in the past, as revealed by the fibrotic/cicatricial lesions found in the liver, and that later on, at 13 months of age, they may have entered in a compensatory phase, in a potential defense mechanism protecting the liver from further damage and progression towards NASH.

To understand if the metabolic dysfunction caused by an HFA background could change the response to a metabolic challenge, animals were also exposed to 10% fructose in the drinking water.

Liver phenotypic observations in fructose fed animals were very redundant with the HFA/STD group induced alterations: FRU animals had increased steatosis and a trend to global fibrosis and no change in ballooning or inflammation. These findings were consistent with findings of other authors, which denote lesions characteristic of the transition from NAFLD to NASH when exposing rats to a long-term high fructose diet (Schultz *et al.*, 2015). However, the HFA background did not seem to alter the effects observed for the metabolic challenge in liver phenotype.

Gene expression after high fructose feeding was also similar to perigestational HFA: all DNL involved genes tended to be downregulated in C/FRU group although only for *mTor* the results obtained reached statistical significance. These findings were not supported by the available literature that overall describe an increase in DNL both with short- and long-term exposure to fructose (Haas *et al.*, 2012, Janevski *et al.*, 2012, Schultz *et al.*, 2015, Ter Horst and Serlie, 2017). However, studies focusing in metabolic disrupted rodent models are contradictory (Leavens *et al.*, 2009), showing no change (Oosterveer *et al.*, 2009) or a decrease (Lee *et al.*, 1994, Brunengraber *et al.*, 2003, Delgado *et al.*, 2009) in lipogenic pathways.

In fact, fructose-rich diets have been associated to the development of insulin resistance (Stanhope *et al.*, 2009) and ectopic lipid deposition (Fisher *et al.*, 2017), as it could be observed in these animals. It is probable that the observed downregulation of lipogenic genes could also represent a compensatory mechanism occurring after major pathological phenotypic alterations such as hepatic steatosis, inflammatory activity and cicatricial lesions.

Since high fructose feeding is a well-characterized metabolic-challenge, obtaining similar effects with HFA exposure corroborates perigestational high folic acid as an effective long-term metabolic challenge.

When studying the interaction between perigestational exposure to HFA and exposure to fructose in DNL genes expression, there is a consistent trend for attenuation of the effect of fructose exposure by HFA background, almost like if perigestational HFA exposure decreased the liver capacity to compensate for the dysfunction in the presence of a metabolic challenge.

The results of this work show that perigestational HFA exposure increased hepatic lipid accumulation. It is well defined that four major pathways are involved in hepatic lipid accumulation: uptake of circulating lipids, DNL, fatty acid oxidation, and lipids escapement (Ipsen *et al.*, 2018). We studied the uptake of lipids

from circulation by evaluating the expression of fatty acid transport proteins which tended to increase by the exposure to HFA during perigestational period, this can partially explain the increased steatosis. DNL was also evaluated and appeared to be decreased in the HFA background which can be a compensatory mechanism to reduce lipidic accumulation in an already ill liver. Studies regarding fatty acid oxidation, and lipids escapement are necessary to understand other processes that may be involved.

These results highlight the importance of FA supplementation with the recommend dose during pregnancy as higher doses may induce hepatic steatosis and may indeed program the offspring for long term metabolic dysfunction.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Journal of Endocrinology

Author guidelines

Editorial Process

Peer review

Submissions are assessed by the Editorial Board and are subject to external peer review using the single blind method whereby the authors are blinded to the identity of the reviewers and editors.

Due to the high volume of submissions received, the Editor-in-Chief and the Editorial Board operate a rapid triage system and only the top 34% of papers undergo full external peer review. This workflow is in place to allow authors to submit to a more appropriate journal with minimal delay.

On average the journal returns a decision on a peer-reviewed paper in 12 days.

Appeals

Authors who feel they have grounds to appeal a rejection decision should send a rebuttal letter to the editorial office, detailing the reasons for the appeal. Rebuttals will be considered by the Editor-in-Chief, often in consultation with the Editorial Board Member who handled the paper. Decisions on appeals are final.

Submission Checklist

Your article

- Structure – Ensure the submission is structured as requested by the journal, and contains all relevant sections. See 'Preparation of Manuscripts' for further details.
- Title page – All submissions must have a title page stating all of the relevant information. See' General' for further details.
- Format – All submissions should follow the journal guidelines for word count, page margins and line numbering. See 'General' for further details.
- Language – Non-native English speakers are encouraged to have their manuscript professionally edited before submission. This is particular key for revised submissions. Click here for full details of our recommended English Language Editing Services.
- Reported data – Data accuracy is crucial. Authors are strongly encouraged to double check all reported data for accuracy and to confirm that all units of measurement are correct and consistent.
- References – Please see 'References' for full details of the journal's required style.
- Graphics - All figures and tables should be presented in a clear and informative manner with accompanying legends.
- Ethical compliance – All articles are required to meet the requirements outlined in our ethical policy. Ensure you have included all relevant ethical approval statements.

Before Submitting

- Approval – Ensure all authors have seen and approved the final version of the article prior to submission. All authors must also approve the journal you are submitting to.
- Open Access – The appropriate Open Access option must be selected on submission. Authors are responsible for ensuring any funder mandates are followed. For further details, please see the Open Access policy.
- Charges – *Journal of Endocrinology* is committed to keeping costs to authors to a minimum, however some charges may apply. Authors are responsible for familiarising themselves with these prior to submission. Full details are available on our Publication charges page.

Uploading your submission

- Author list – All authors must be listed on the title page and entered on the ScholarOne Manuscripts submission in the correct order. Ensure all author email addresses provided are valid. Author information entered into ScholarOne Manuscripts will be used to generate PubMed listings for published papers.
- Cover Letter - This letter should introduce your paper and outline why your work is important and suitable publication at this time.
- File formats – Ensure all files are in the correct format for revised submissions. See 'General' for further instructions.
- Figures and tables – Ensure all figures and table files are present and correct, and that they display clearly in the PDF proof.

Preparation of Manuscripts

General

Manuscripts should:

- Be concise and clear.
- Be limited to 5000 words for Research submissions. For information on other manuscript types please see the

relevant section below.
- Display the word count on the title page.
- Contain no more than 10 figures and 60 references as recommended by the journal.

- Use double line spacing throughout (including reference list and figure legends), and contain continuous line numbering down the left-side of each page.
- Define all abbreviations when first mentioned.
- Be submitted in the correct file type, ie. Main document in an editable Word format.
- Be written in either UK or US English.
- Contain a title page.

Please be aware that the combined size of your files should not exceed **40 MB**.

Accepted file types:
- For article text: txt, doc, docx, tex, (pdf [first submissions only])*
- For figures: eps, tiff, jpg, pdf

*We are unable to accept PDF files for article text for revised manuscripts.

Changes within revised manuscripts should be highlighted using the highlighter function or coloured text, and should be accompanied by a full response letter to editor and reviewer comments.

Research
All research submissions should be formatted in the following sections:

1) Title Page

Include a separate title page with:
- Title (maximum 85 characters)
- All authors names and full addresses
- Corresponding author's postal and email address
- A short title (maximum 46 characters, including spaces)
- A minimum of four keywords describing the manuscript
- Word count of the full article, excluding references and figure legends

2) Abstract

The abstract should be a single paragraph of not more than 250 words, clearly stating the objective of the study or review, the methods used (where applicable), and summarizing results and conclusions. Avoid abbreviations and references in this section.

3) Introduction

The introduction should set the study in context by briefly reviewing relevant knowledge of the subject; follow this with a concise statement of the objectives of the study.

4) Materials and methods

Provide sufficient information for other workers to repeat the study. If well-established methods are used give a reference to the technique and provide full details of any modifications.
- Include the source of chemicals, reagents and hormones and give the manufacturer's name and location (town, country) in parentheses.
- Give the generic name, dose and route of administration for drugs.
- Specify the composition of buffers, solutions and culture media.
- Use SI symbols, give concentrations in mol/L and define the term % as w/v or v/v for all solutions. For international units use IU (U should be used for enzyme activity).
- Specify the type of equipment (microscopes/objective lenses, cameras, detectors) used to obtain images.
- Specify any image acquisition software used, and give a description of specialized techniques requiring large amounts of processing, such as confocal, deconvolution, 3D reconstructions, or surface and volume rendering.

5) Results

6) Discussion

7) Declaration of interest, Funding and Acknowledgements

Declaration of interest
Actual or perceived conflicts of interest for all authors must be declared in full.

Please either (a) declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported; or (b) fully declare any financial or other potential conflict of interest.

Conflicts of interest include, but are not limited to:
- Employment and consultancies
- Grants, fees and honoraria
- Ownership of stock or shares
- Royalties
- Patents (pending and actual)
- Board membership
-

Funding
Please detail all of the sources of funding relevant to the research reported in the following format:
This work was supported by the Medical Research Council (grant numbers xxxx, yyyy); the Wellcome Trust (grant number xxxx); and Tommy's Baby charity (grant number xxxx).

Where research has not been funded please state the following:
This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement (optional)
Please include a statement specifying the contribution of each co-author.

Acknowledgements
Please be as brief as possible.

8) References

All references cited in the text must be included in the reference list and *vice versa*. However, if a reference consists of only a web address do not include it in the reference list but cite it in the text, giving the date the page was accessed.

Unpublished work
Any unpublished work (personal communications, manuscripts in preparation and manuscripts submitted but not yet accepted for publication) must be referred to in the text and not listed in the references.

Give the full list of authors, including their initials. For example:
(A Stone, J Brown & M R Smith, unpublished observations)
(J Brown, personal communication)

Articles accepted for publication but not yet published may be listed as 'in press' in the reference list, using the current year as the publication year. If an 'in press' article is included in the Accepted Preprint service or a similar scheme, then the Digital Object Identifier (DOI) should be included; otherwise, provide a copy of the article as a supplementary file for reviewing purposes.

In the text
Cite references in the text using the authors' names and publication year. Use *et al.* for articles with more than two authors. Where there are several citations, list them in chronological order.

In the reference list

List references in alphabetical order. Give articles by the same author in the order:

- Single author
- Two authors alphabetically according to the name of the second author
- Three or more authors chronologically, with a, b and c etc. for articles published in the same year, in the order in which they are cited in the text

List a maximum of ten authors. Where there are more than ten authors, list the first ten and then use *et al*.

Reference in the following format:
See RH, Calvo D, Shi Y, Kawa H, Luke MP & Yuan Z 2001 Stimulation of p300-mediated transcription by the kinase MEKK1. *Journal of Biological Chemistry* **276** 16310–16317.

Harvey SS 1975 Hypnotics and sedatives. The barbiturates. In *The Pharmacological Basis of Therapeutics*, edn 5, pp 102–123. Eds LS Goodman & A Gilman. New York: Macmillan Publishing Co.

EndNote
Please use Harvard style.

9) Tables

Tables should be concise. Tables too large for print publication should be submitted as supplementary data.

- Number tables in the order they are cited in the text
- Include a title – a single sentence at the head of the table that includes the name of the organism studied
- Use footnotes to provide any additional explanatory material, cross-referenced to the column entries
- Give a short heading for each column
- Do not use internal horizontal or vertical lines, colour or shading
- Explain all abbreviations used in the table in the footnotes

10) Figures

The journal has produced digital image guidelines in order to clarify the standards expected by the journal. All submitted digital images must adhere to these guidelines.

Colour figures will be published online at no charge to the author. Publication of colour figures in the print version will incur a charge that must be paid before publication. Please note the option to print in greyscale is subject to editorial approval, if the meaning of your image is unclear you will be asked to print in colour and a charge will apply.

- Number figures in the order they are cited in the text
- Include legends to all figures, giving the figure number, keys to any symbols used, the name of the organism studied, the names of any statistical tests used and the probability levels used for comparisons
- Label figure sections as A, B etc in the top left-hand corner
- Use Arial or a similar sans-serif font for text labels
- Do not enclose figures in boxes
- Indicate magnification by a scale bar in the bottom right-hand corner of the image and give the measurement in the legend
- Use the preferred symbols of closed and open circles, squares and triangles. Ensure that symbols are large enough to be read clearly when the figure is reduced for publication
- Use Courier or a similar non-proportional font for amino acid, DNA, RNA and PCR primer sequences and highlight sections of homology between sequences with grey shading

File types and resolution
Journal of Endocrinology is committed to publishing high quality figures.

EPS or TIFF files are preferred. Files should be exported in Illustrator compatible format. Avoid using PowerPoint or Word files for figures.

Additional information regarding the submission of figures can be found here.
Line images/graphs

- File types: EPS, TIFF, high-resolution PDF, AI (Adobe Illustrator)
- Resolution at final published size: 1200 dpi

Half-tone (greyscale) images

- File types: TIFF, high-resolution PDF, JPEG
- Resolution at final published size: 600 dpi

Colour images

- File types: TIFF, high-resolution PDF, JPEG. EPS or AI files can be used for graphical data and illustrations that don't include photographs
- Resolution at final published size: 300 dpi
- Colour format: CMYK (not RGB)

Journal of Endocrinology also accepts the following manuscript types. For full details please see the 'scope' page.

Reviews

The format of review articles is more fluid but should include the following:

1. Title page
2. Abstract
3. Conclusions
4. Declaration of interest, Funding, Author contributions statements (where appropriate)
5. References
6. Figure legends
7. Figures/ tables

Review submissions should be limited to 6000 words. We recommend a maximum of 60 references for review articles, with 2--6 figures and tables. Original summary diagrams and illustrations of proposed models (in colour where appropriate) are encouraged. Line drawings may be redrawn. Boxes can be used to separate detailed explanations and background information from the main part of the text.
For further information on what is include in each of these sections, please see the 'Manuscript Preparation' section above.

Editorials

All Editorials must be a maximum 1500 words (including references, legends and tables) and 10 references. If you would like to submit an article for consideration please submit a proposal to the editorial office.

Supplementary Data

Supplementary data too large for print publication or exceeding the bounds of the manuscript may be submitted for online publication.

Supplementary data files intended for online publication should be submitted online via ScholarOne Manuscripts as 'Supplemental File for Review', and referred to as supplementary data in the text:

(Supplementary Table 1)
(Supplementary Figures 1 and 2)

Supplementary information will be reviewed as part of the manuscript, evaluated for its importance and relevance and, if accepted, will be referenced in the text of the article, directing readers to the website. All submissions with supplementary data listed as 'Supplementary File for Review' will be reviewed under the assumption that the data will be published as part of the final article.

There is a charge for publication of supplementary data. Should authors not wish to publish their supplementary data, they must notify the editorial office prior to acceptance.

Editorial Policy

Human Subjects Research

Human subjects

Authors must ensure research involving human subjects complies with the Declaration of Helsinki.
Authors must include a statement that consent has been obtained from each patient after full explanation of the purpose and nature of all procedures used. For research requiring ethics committee approval, please include a statement to this effect in the manuscript. Also indicate whether patient consent was obtained in line with the below policy. We will be unable to accept research papers without this statement.

Animal studies

Experiments with animals must be performed in accordance with international, national and institutional requirements. Include a statement that investigations have been approved by the local ethical committee, along with the following.

- Give the full binomial Latin names for all experimental animals other than common laboratory animals
- State the breed or strain and source of animals, and give details of age, weight, sex and housing
- Detail the procedures and anaesthetics used, including doses given

Articles will only be considered if the procedures used are clearly described and conformed with the international and national legal and ethical requirements, as well as the requirements outlined by the institution in which the work took place. A statement identifying the committee approving the study must also be included in the Methods section.
Authors are encouraged to refer to the ARRIVE guidelines, and in particular the checklist within them, when preparing manuscripts detailing animal experiments.
Editors reserve the right to request further information on the exact procedures and ethical approval obtained as part of the review process. Papers may be rejected on ethical grounds should the editors feel the study does not adequately meet current international guidelines for humane research.

Experiments with genetically engineered mice

In inbred mice, genetic strain effects can have significant effects on phenotype. Because of this the following controls for experiments with genetically-manipulated mice should be used: parental inbred strain, or wild-type littermates.

Cell Lines

In general, studies that are based on observations performed in a single cell line will not be considered for publication if other lines of the same general lineage and characteristics are available. If at all possible, observations should be replicated in multiple cell lines.

Authentication of cell lines

We require that all cell lines are authenticated for correct origin. Specifically, the author should include the following information supporting the authentication of lines:

1. Source of cell lines. Gifts of cell lines from individuals will not be acceptable
2. Please state what the method of authentication is. For example, ATCC uses STRS analysis
3. State the passage number(s) of cell lines used for the experiments described in the submission. Unless the research is specifically about senescence, lines >35 passages would not be acceptable

Genetic association case/control studies

The Society for Endocrinology has produced guidelines for genetic association case/control studies in order to ensure they are methodologically sound and readers can fully assess their significance.

All Society for Endocrinology journals require data from such studies to be deposited in public databases (eg MIAME or GEO) or the manuscript will not be published. *Journal of Endocrinology* supports the public dissemination of gene expression data.

Gene and protein nomenclature

Wherever possible, manuscripts must be prepared in accordance with approved gene nomenclature.

- In gene and protein symbols, substitute Greek letters with the corresponding roman letter, e.g. TGFBR2 not TGFβR2
- Avoid hyphens unless they are part of the approved symbol, e.g. IGF1 not IGF-1
- Use arabic rather than roman numerals, e.g. BMPR2 not BMPRII

Follow species-specific formatting standards as follows:

Mice and rats

- Gene symbols should be in italics with only the first letter capitalised, e.g. Sox2
- Protein designations should be the same as the gene symbols except that all letters should be capitalised and in roman (i.e. not italicised), e.g. SOX2
- Please use symbols approved by the International Committee on Standardized Genetic Nomenclature for Mice and the Rat Genome and Nomenclature Committee, which can be queried at the MGI website

Humans, non-human primates and domestic species

- Gene symbols should be in italics with all letters capitalised, e.g. SOX2
- Protein designations should be the same as the gene symbols but not italicised, e.g. SOX2
- Please use symbols approved by the HUGO Gene Nomenclature Committee (HGNC)

Fish

- Gene symbols should be in italics with all letters in lower case, e.g. sox2
- Protein designations should be the same as the gene symbols but not italicised and with the first letter capitalised, e.g. Sox2
- Please use symbols approved by the Zebrafish Nomenclature Committee (ZNC), which can be queried at the ZFIN website

Digital image guidelines

Recognizing that the inappropriate use of computer software for digital image analysis and processing can raise concerns, the journal has produced the following requirements for the representation of research data:

- No specific feature within an image may be enhanced, obscured, moved, removed, or introduced
- Adjustments of brightness, contrast, or colour balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Any such adjustments should be applied to the entire image
- Threshold manipulation, expansion or contraction of signal ranges and the altering of high signals should be avoided
- The legend to a digital image should state if and what digital modifications were applied

The following guidelines apply to digital images that that result from gel electrophoresis and blotting procedures:

- Band intensity should be quantified from several independent experiments. If only a "typical" experiment is shown in the figure, authors should be prepared to provide unprocessed images of gels or blots at the request of the Editor-in-Chief.
- Extensively cropped images are not acceptable. Images can be cropped to enhance clarity of presentation, but should always contain at least two markers (one with a smaller, one with a larger molecular size than the band of interest) with their molecular sizes indicated.
- Producing spliced images by combining lanes from gels or blots from different experimental runs should be avoided. A lane containing markers should be on the same gel for each run. If spliced images are presented, the vertical lanes obtained from gels or blots from different experimental runs should be clearly demarcated with lines.
- As the validity of immunoblots relies heavily on antibody specificity, an appropriate control (tissue from knockout mice or protein knockdown in cell lines) must be included, or alternatively a reference should be given in the methods section referring to such a control (Saper 2009; Burry 2011).
- The reuse of images of loading controls from other experiments or previous publications is unacceptable.

References

Burry RW 2011 Controls for immunocytochemistry: An update. *Journal of Histochemistry & Cytochemistry* **59** 6–12. (doi:10.1369/jhc.2010.956920)
Saper CB 2009 A guide to the perplexed on the specificity of antibodies. *Journal of Histochemistry & Cytochemistry* **57** 1–5. (doi:10.1369/jhc.2008.952770)

Microscopy

Microscope images should be made available to referees in images that are at least 300 dpi at the size which they will be published. 'Pseudo-colouring' and nonlinear adjustment (for example 'gamma changes') are only allowed if unavoidable and must be disclosed.

Statistical analysis

It is the author's responsibility to document that the results are reproducible and that the differences found are not due to random variation. No absolute rules can be applied but, in general, quantitative data should be from no fewer than three replicate experiments. Appropriate statistical methods should be used to test the significance of differences in results. The term 'significant' should not be used unless statistical analysis was performed, and the probability value used to identify significance (e.g. $P < 0.05$) should be specified.

When several t-tests are employed, authors should be aware that nominal probability levels no longer apply. Accordingly, the multiple t-test, multiple range test, or similar techniques to permit simultaneous comparisons should be employed. Also, in lieu of using several t-tests, it is often more appropriate to utilize an analysis of variance (ANOVA) to permit pooling of data, increase the number of degrees of freedom, and improve reliability of results. Authors should use appropriate nonparametric tests when the data depart substantially from a normal distribution.

In presenting results of linear regression analyses, it is desirable to show 95% confidence limits.

When data points are fitted with lines, specify the method used for fitting (graphical, least squares, computer program). If differences in slopes and/or axis intercepts are claimed for plotted lines, these should be supported by statistical analysis.
Give sufficient details of the experimental design and analysis so that the reader can assess their adequacy and validity for testing the hypotheses of interest.
In particular:

- Describe the numbers of experimental units used and the way in which they have been allocated to treatments
- Justify the omission of any observations from the analysis
- Describe methods of analysis precisely and state any necessary assumptions, as these may affect the conclusions that can be drawn from the experiment

Your article may be sent to the Statistical Advisor for comments.

Preprint and data repositories

Preprint repositories

A preprint is a version of the article prior to submission to the journal for peer review, and has not been copyedited or typeset.
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Depositing data in public databases

Authors are strongly encouraged to deposit data sets in appropriate public databases. Authors should include the relevant database identifiers and accession numbers for deposited sequences within the manuscript using the following format: Database: xxxx, e.g: GEO: GSE6364. Authors are also required to provide the URL for the sequence(s).
Below is a list of public databases:

- Addgene
- Array Express
- ClinVar
- DNA Databank of Japan
- dbGaP
- dbSNP
- dbVar
- ENA's Sequence Read Archive
- GenBank
- NCBI Trace and Short-Read Archive
- PeptideAtlas
- PRIDE
- Protein DataBank
- PubChem
- Uniprot
- Gene Expression Omnibus (GEO)

This list is not exhaustive. Please contact the editorial office if you have a query about relevant databases.

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