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# Maternal Fructose Intake Increases Liver $H_2S$ Synthesis but Exacerbates its Fructose-Induced Decrease in Female Progeny

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**Scope: Fructose intake from added sugars correlates with the epidemic rise in metabolic syndrome and cardiovascular diseases (CVD). However, consumption of beverages containing fructose is allowed during gestation. Homocysteine (Hcy) is a well-known risk factor for CVD while hydrogen sulfide ($H_2S$), a product of its metabolism, has been proved to exert opposite effects to Hcy.**

**Methods and results: First, it is investigated whether maternal fructose intake produces subsequent changes in Hcy metabolism and $H_2S$ synthesis of the progeny. Carbohydrates are supplied to pregnant rats in drinking water (10% wt/vol) throughout gestation. Adult female descendants from fructose-fed, control or glucose-fed mothers are studied. Females from fructose-fed mothers have elevated homocysteinemia, hepatic $H_2S$ production, cystathionine $\gamma$-lyase (CSE) (the key enzyme in $H_2S$ synthesis) expression and plasma $H_2S$, versus the other two groups. Second, it is studied how adult female progeny from control (C/F), fructose- (F/F), and glucose-fed (G/F) mothers responded to liquid fructose and compared them to the control group (C/C). Interestingly, hepatic CSE expression and $H_2S$ synthesis are diminished by fructose intake, this effect being more pronounced in F/F females.**

**Conclusion: Maternal fructose intake produces a fetal programming that increases hepatic $H_2S$ production and, in contrast, exacerbates its fructose-induced drop in female progeny.**

## 1. Introduction

It has been well-established that metabolic events during pre- and postnatal development modulate metabolic disease risk in later life.[1] Maternal feeding conditions is the most important event affecting the health of offspring.[2] Fructose is used as added sugar in the form of sucrose or high fructose corn syrup to sweeten a wide array of processed foods and sugary drinks. Unfortunately, fructose has frequently been linked to obesity, metabolic syndrome, and cardiovascular diseases (CVD).[3] Experimental studies have shown that fructose can provoke many features of metabolic syndrome in rats, whereas this is not the case with glucose.[3] In fact, we have previously shown that maternal fructose intake provokes in adult male, but not female, offspring an impaired insulin signaling and hyperinsulinemia.[4] Interestingly, female progeny born of fructose-fed mothers presented an exacerbated response to liquid fructose intake when adults displaying hypertriglyceridemia and hepatic steatosis,[5] while these risk factors for metabolic syndrome were not found in females from control or glucose-fed mothers.

Thus, although a connection between a high consumption of fructose-containing beverages and the global epidemic of obesity and metabolic syndrome could exist,[6,7] ingestion of these beverages and fruit juices is still permitted during gestation. Possibly, the reason for this inconsistency is that the debate about the relationship between fructose and CVD remains alive. There are studies reporting that fructose causes metabolic syndrome while other studies have not found that connection.[8–11]

CVD is one of the most prevalent diseases in humans and, in fact, it remains as the leading cause of death around the world.[12] However, most experimental studies or clinical trials are carried out in males and/or men, studies in females and/or women still being quite scarce. Interestingly, disturbances such as diabetic dyslipidemia and CVD associated with diabetes mellitus type 2 seem to be more prevalent in females.[13] Related to this, women, but not men, exhibit an association between fructose consumption and an increased risk of type 2 diabetes mellitus.[14] Moreover, a recent report from the Center for Chronic Disease

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Prevention and Health Promotion has found that heart disease was the leading cause of death in women in the USA in 2017.

Homocysteine (Hcy) is a non-protein amino acid that has been recognized as a risk factor for hypertension, vascular dysfunction, CVD, etc., when it is elevated in plasma.[15,16] In healthy conditions, plasma homocysteine levels are maintained by elimination of the excess via the remethylation pathway to regenerate methionine and via the transsulfuration pathway to generate cysteine and hydrogen sulfide ($H_2S$).[16] Curiously, $H_2S$ is a water-soluble molecule that acts as a gasotransmitter[17] in which there has been increasing interest since it shows neuroprotective and cardioprotective benefits.[16] Thus, whereas Hcy is considered a major risk factor for atherosclerosis, $H_2S$ is a molecule that seems to protect against the pathogenesis of atherosclerosis.[18] Interestingly, in a previous report, we detected decreased plasma folic acid levels in females from fructose-fed dams, in comparison to the offspring from control and glucose-fed mothers[19] and, in general, plasma folate is inversely related to plasma Hcy.[15,20]

The liver plays a key role in Hcy and $H_2S$ metabolism and is sensitive to nutrient imbalances.[16] For example, high-fat diets (HFD) induce features characteristic of non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of metabolic syndrome, such as steatosis, oxidative stress, and inflammation.[16] However, there are controverted results regarding the effects of HFD in Hcy metabolism and $H_2S$ synthesis. Thus, Hwang et al. have reported that a HFD stimulates Hcy metabolism through the transsulfuration pathway, yielding $H_2S$ and decreasing plasma Hcy.[16] However, Peh et al. have reported that HFD reduces hydrogen sulfide metabolism.[21] Curiously, some of these alterations observed in metabolic syndrome (steatosis, oxidative stress, etc.) have also been found by others, including ourselves, in fructose feeding animal models.[4,5,22,23] However, direct effects of fructose in Hcy and $H_2S$ metabolisms have hardly been documented[24] and moreover, studies regarding the effects of maternal fructose in the $H_2S$ and Hcy pathways of the progeny are lacking.

In order to investigate whether maternal fructose programmes Hcy metabolism and $H_2S$ synthesis in female progeny, parameters related to these routes, and some of the risk factors for metabolic syndrome were measured in female rats from control, fructose- or glucose-fed dams. Subsequently, for the purpose of checking whether adult female progeny born of fructose-fed mothers showed a different response to liquid fructose intake in comparison to females from control and glucose-fed mothers, we studied both homocysteine and $H_2S$ metabolisms in liver and plasma of adult female rats born from control, fructose- or glucose-fed mothers, after themselves receiving fructose as a liquid solution (10% wt/vol in drinking water) for 3 weeks.

## 2. Results

### 2.1. Maternal Fructose Increases Hepatic $H_2S$ Production in Female Progeny

In a recent report,[19] we found that maternal fructose intake provoked a decrease in plasma folic acid levels in adult female progeny (39.6 ± 1.7; 27.6 ± 1.4; and 36.2 ± 7.2 ng $mL^{-1}$ for female offspring from control, fructose- and glucose-fed dams, respectively; $p < 0.05$, for progeny of fructose-fed mothers vs control group). It has been reported that plasma folate and plasma homocysteine generally display an inverse correlation.[20] Therefore, in the present study, we determined plasma homocysteine levels of the progeny from control, fructose-, and glucose-fed mothers. As expected, female progeny born of fructose-fed mothers tended to display an increase in plasma homocysteine levels versus the other two groups (**Figure 1**A), the difference being almost significant ($p = 0.075$) in the progeny of fructose-fed mothers versus control group.

Homocysteine is a non-proteinogenic amino acid and its levels are regulated through two metabolic pathways: transsulfuration and remethylation.[18] The remethylation pathway is governed by two enzymes: methionine synthase (MTR) and betaine:homocysteine methyltransferase (BHMT). As shown in Figure 1, female progeny from fructose-fed mothers displayed lower BHMT expression, and this difference turned out to be significant versus females from glucose-fed dams (Figure 1B). There was no difference in the MTR expression between the three experimental groups (Figure 1C). Nevertheless, the lower folic acid levels found in the offspring from fructose-supplemented mothers would indicate a lower remethylation of homocysteine using folic acid as a methyl donor in this group in comparison to the other two groups.

Regarding transsulfuration, this pathway is carried out by two enzymes: cystathionine $\beta$-synthase (CBS) and cystathionine $\gamma$-lyase (CSE). As shown in Figure 1, gene expression of both enzymes was significantly augmented in females from fructose-fed mothers versus progeny of control and glucose-fed mothers (Figure 1D, for CBS; Figure 1E, for CSE). Therefore, the transsulfuration pathway was clearly activated by maternal fructose intake, since these animals, after delivery, had received no sugar additive in the drinking water.

$H_2S$ is produced in the transsulfuration pathway and, accordingly, has been related to homocysteine metabolism. Therefore, plasma $H_2S$ levels were measured and, as shown in **Figure 2**A, female progeny born of fructose-fed mothers tended to display an increase versus the other two groups, although without becoming significantly different. Interestingly, $H_2S$ production in liver, the organ where this gasotransmitter is mainly generated,[25] was significantly increased in females of fructose-fed mothers versus control progeny or females from glucose-fed dams (Figure 2B), in agreement with the findings found for CBS and CSE expression (Figures 1D,E).

It has been reported that upregulation of the transsulfuration pathway and the subsequent $H_2S$ production might play an adaptive role against oxidative stress.[16] In agreement with this, females from fructose-fed mothers displayed lower lipid peroxidation (measured as malondialdehyde, MDA, levels) in the liver than the other two groups, becoming significant in comparison to female progeny of control mothers (Table S1, Supporting Information). Curiously, while lipid oxidation was reduced in females from fructose-fed dams, there was a rise in protein oxidation (measured as protein carbonyls) in offspring from carbohydrate-fed mothers, this being significant between female progeny of control and fructose-supplemented mothers (Table S1, Supporting Information). However, the levels of hepatic methylglyoxal, an inducer of oxidative stress and protein carbonylation, were not different. Furthermore, whereas one of the

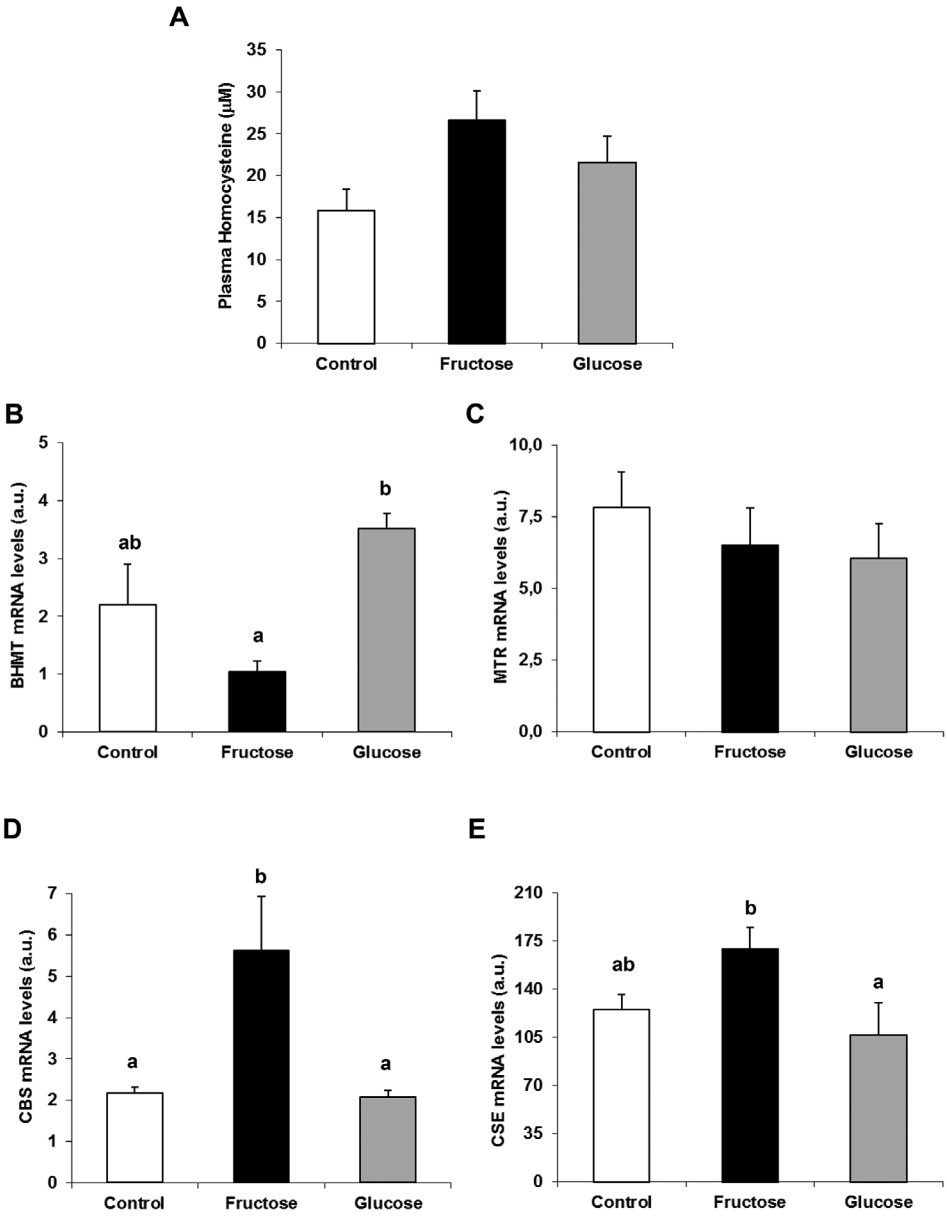


**Figure 1.** Fructose in pregnancy affects hepatic Hcy metabolism in adult female progeny. A) Plasma homocysteine levels and relative hepatic levels of specific mRNA for both remethylation pathway genes: B) BHMT, C) MTR; and transsulfuration pathway genes: D) CBS and E) CSE of 261-day-old female progeny from control (empty bar), fructose-fed (black bar), and glucose-fed (grey bar) pregnant rats. Relative target gene mRNA levels were measured by real time PCR, as explained in Section 4, normalized to Rps29 levels and expressed in arbitrary units (a.u.). Data are means ± S.E. from 5–6 litters. Values not sharing a common letter are significantly different ($p < 0.05$). Hcy: homocysteine; BHMT: betaine-homocysteine methyltransferase; MTR: methionine synthase; CBS: cystathionine $\beta$-synthase; CSE: cystathionine $\gamma$-lyase.

major antioxidant buffers in the liver (reduced glutathione, GSH) was unchanged between the three groups (Table S1, Supporting Information), the activity of one of the most important hepatic antioxidant enzyme (manganese superoxide dismutase, SOD2) was augmented in females from fructose-fed mothers, being significantly different versus progeny from glucose-fed dams (Table S1, Supporting Information). In agreement with these results, hydrogen sulfide has been reported to ameliorate oxidative stress in mouse cardiomyocytes by upregulating SOD2.[26] In fact, concentration of an important nonenzymatic prooxidant in liver (uric acid) tended to be diminished in females from fructose-fed mothers.

Many factors have been discovered to regulate CSE expression and activity (for a review, ref. [27]). A number of transcription factors, including Sp1, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), activating transcription factor 4 (ATF4), and Elk1, regulate CSE transcription through direct or indirect binding with CSE promoter. However, mRNA gene expression of ATF4, TNF$\alpha$, and calmodulin (a protein related to the calcium levels) showed no differences between the three groups (**Table 1**). TNF$\alpha$ and

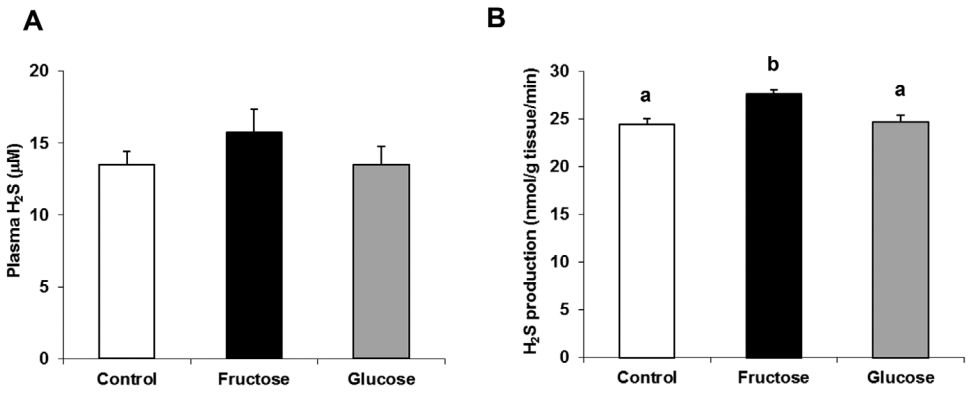


**Figure 2.** Fructose in pregnancy influences plasma $H_2S$ by affecting its hepatic production. A) Plasma $H_2S$ levels and B) hepatic $H_2S$ production of 261-day-old female progeny from control (empty bar), fructose-fed (black bar) and glucose-fed (grey bar) pregnant rats. Data are means ± S.E. from 5–6 litters. Values not sharing a common letter are significantly different ($p < 0.05$).

**Table 1.** Molecular regulators of CSE expression and $H_2S$ production. Liver gene expression (mRNA) of transcription factors and/or their target genes of 261-day-old female progeny from fructose- or glucose-supplemented and control mothers.

| | Control | Fructose | Glucose |
|---|---|---|---|
| ATF4 | 1.12 ± 0.19 | 1.61 ± 0.29 | 1.26 ± 0.15 |
| TNF$\alpha$ | 1.04 ± 0.38 | 0.99 ± 0.04 | 0.62 ± 0.25 |
| Calmodulin 3 | 1.07 ± 0.11 | 1.29 ± 0.08 | 1.13 ± 0.06 |
| HDAC3 | 2.10 ± 0.14 | 2.38 ± 0.10 | 2.35 ± 0.19 |
| XBP1u | 0.59 ± 0.04$^{a)}$ | 0.86 ± 0.02$^{b)}$ | 0.61 ± 0.17$^{ab)}$ |
| SLC6A6 | 5.75 ± 0.69$^{a)}$ | 10.42 ± 0.63$^{b)}$ | 6.03 ± 1.35$^{a)}$ |
| SLC6A12 | 3.00 ± 0.32 | 3.98 ± 0.47 | 2.44 ± 0.66 |
| Aldose reductase | 12.7 ± 0.8 | 16.3 ± 1.3 | 13.5 ± 2.5 |
| Sorbitol dehydrogenase | 7.67 ± 0.71$^{a)}$ | 11.93 ± 1.01$^{b)}$ | 8.77 ± 1.23$^{ab)}$ |

Data are M ± S.E., from 5–6 litters. Relative target gene mRNA levels were measured by real time PCR, as explained in Section 4, and normalized to Rps29 levels and expressed in arbitrary units (a.u.). Different letters indicate significant differences between the groups ($p < 0.05$). TNF$\alpha$: Tumor necrosis factor alpha; ATF: Activating transcription factor; HDAC: Histone deacetylase; XBP1u: unspliced X-box-binding protein 1; SLC: Solute carrier.

calcium seem to regulate CSE gene expression by stimulating binding of Sp1 to the CSE promoter (ref. [27] and references therein). On the other hand, Nrf2 is a transcription factor involved in antioxidant responses and promotes transcription of genes, including the CSE gene.[27] It has been reported, and we have recently been able to confirm this,[28] that the interaction between unspliced X-box-binding protein 1 (XBP1u) and histone deacetylase 3 (HDAC3) modulates oxidative stress by stabilizing Nrf2 transcription factor.[29] Notably, although HDAC3 gene expression did not display any differences among the three groups, XBP1u mRNA levels were clearly augmented in liver of females from fructose fed mothers versus the other two groups (Table 1), coincident with the increase observed in the SOD2 activity (Table S1, Supporting Information). Moreover, several putative binding sites for family members of nuclear factor of activated T cells (NFAT) have recently been identified in the CSE promoter.[30] In accordance with this, the mRNA levels for several NFAT5 target genes (SLC6A6, SLC6A12, aldose reductase, and sorbitol dehydrogenase) were augmented in female rats from fructose-fed dams, becoming significant in comparison to female progeny of control mothers for SLC6A6 and sorbitol dehydrogenase genes (Table 1).

Therefore, maternal fructose intake provokes a clear induction of the hepatic transsulfuration pathway with a subsequent increased production of $H_2S$ in adult female progeny, whereas descendants from glucose-fed or control mothers did not exhibit any of these modifications.

### 2.2. Maternal Fructose Exacerbates Fructose-Induced Repression of Hepatic $H_2S$ Production in Female Progeny

Bearing in mind the metabolic changes observed in female progeny born from fructose-supplemented mothers and in order to discover if this phenotype was conserved or reversed by a short liquid fructose-feeding period (3 weeks), we subjected female progeny from control, fructose-, and glucose-fed mothers to a fructose liquid solution and determined if the maternal fructose intake influences the adult offspring's response to fructose.

As shown in **Figure 3**A, plasma homocysteine was not affected by fructose ingestion, although it tended to be diminished in progeny from fructose- and glucose-mothers. Curiously, plasma folic acid levels in adult female progeny were decreased by fructose intake independently of whether they were born from control, fructose-fed, or glucose-fed mothers (43.1 ± 3.7; 20.2 ± 1.6; 23.7 ± 2.2; and 24.4 ± 3.6 ng $mL^{-1}$ for C/C, C/F, F/F, and G/F, respectively; $p < 0.05$, for the three groups of fructose-fed rats vs control (C/C) group). Regarding the gene expression of enzymes that regulate the homocysteine and folic acid levels, BHMT mRNA levels tended to be increased in the F/F group, although without becoming significantly different (Figure 3B); and MTR mRNA levels were not affected by fructose intake, although in the C/F group, it seems to be diminished in comparison with the other three groups (Figure 3C). Interestingly, fructose consumption did affect the transsulfuration pathway in female rats. In fact, whereas CBS gene expression was significantly increased by fructose ingestion only in the progeny from glucose-fed mothers (Figure 3D), CSE expression was

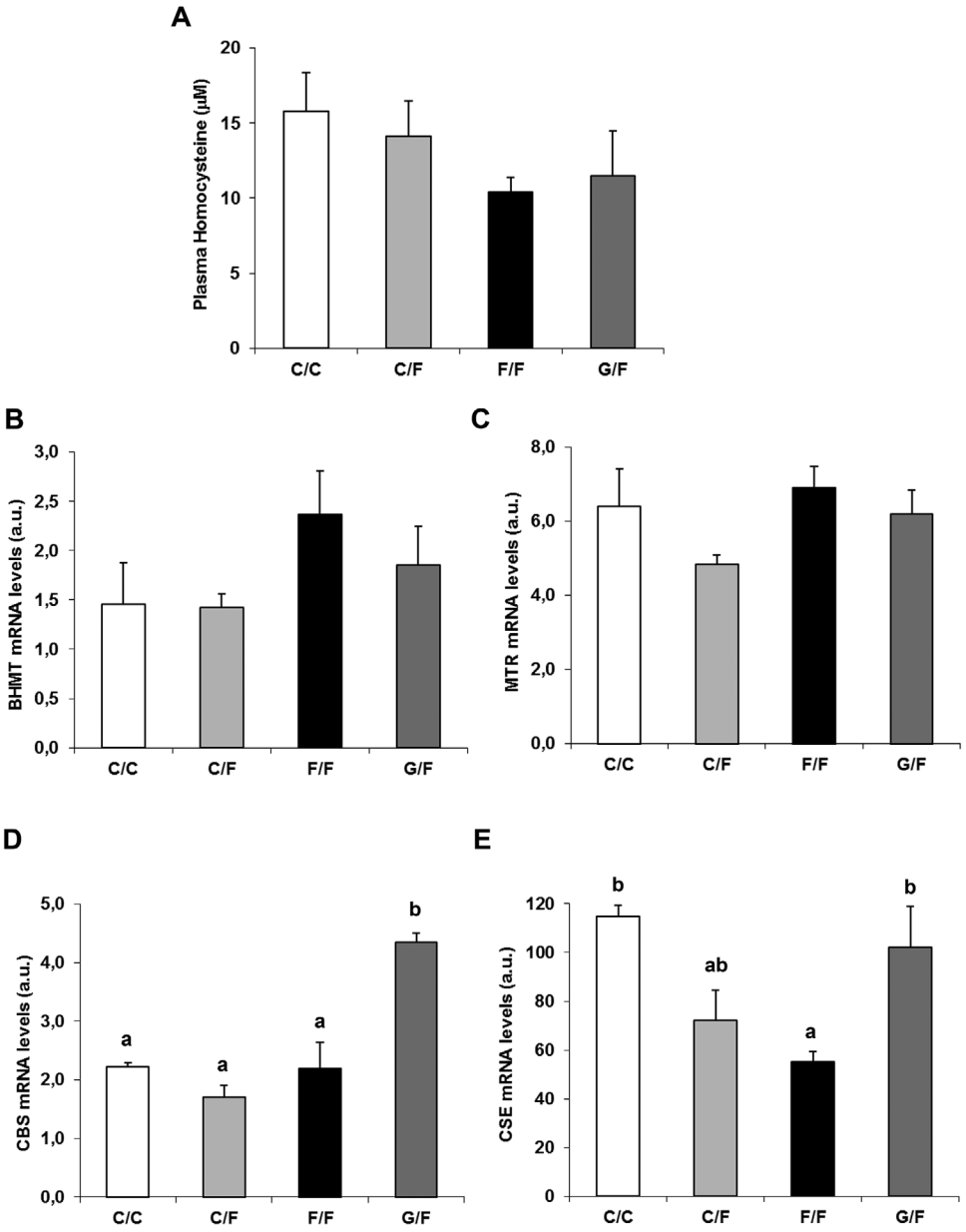


**Figure 3.** Liquid fructose in gestation exacerbates fructose-induced repression of CSE expression in adult female progeny. A) Plasma homocysteine levels and relative hepatic levels of specific mRNA for both remethylation pathway genes: B) BHMT, C) MTR; and transsulfuration pathway genes: D) CBS and E) CSE. Liver (mRNA) expression of fructose-fed female adult progeny from control (C/F, light grey bar), fructose- (F/F, black bar), and glucose-supplemented (G/F, dark grey bar) mothers. C/C: Control 261-day-old female offspring from control pregnant rats (empty bar, C/C). Relative target gene mRNA levels were measured by real time PCR, as explained in Section 4, normalized to Rps29 levels and expressed in arbitrary units (a.u.). Data are means ± S.E. from 5–6 litters. Values not sharing a common letter are significantly different ($p < 0.05$). BHMT: betaine-homocysteine methyltransferase; MTR: methionine synthase; CBS: cystathionine $\beta$-synthase; CSE: cystathionine $\gamma$-lyase.

repressed in fructose-fed rats and this diminution was clearly maternal-intake dependent (Figure 3E). Thus, fructose-induced reduction in CSE expression was more effective in progeny from fructose-fed mothers, becoming significantly different effect compared to rats from glucose-mothers and control (C/C) group.

It is well-known that mainly CSE, and CBS in minor extension, regulate the $H_2S$ production in liver.[31] Therefore, $H_2S$ synthesis was measured in liver extracts from the four experimental groups, using both homocysteine and cysteine as substrates. When cysteine is used, $H_2S$ is produced by the activity of both CSE and CBS, whereas homocysteine is substrate only for CSE.[31] Thus, in accordance with the findings found for CSE expression (Figure 3E), fructose ingestion diminished hepatic $H_2S$ production and this effect was maternal-intake dependent. As shown in **Figure 4**A, a fructose-induced reduction of $H_2S$ generation was observed in the three experimental groups, being more pronounced in progeny from fructose-fed mothers. These changes were found to be significantly different for F/F

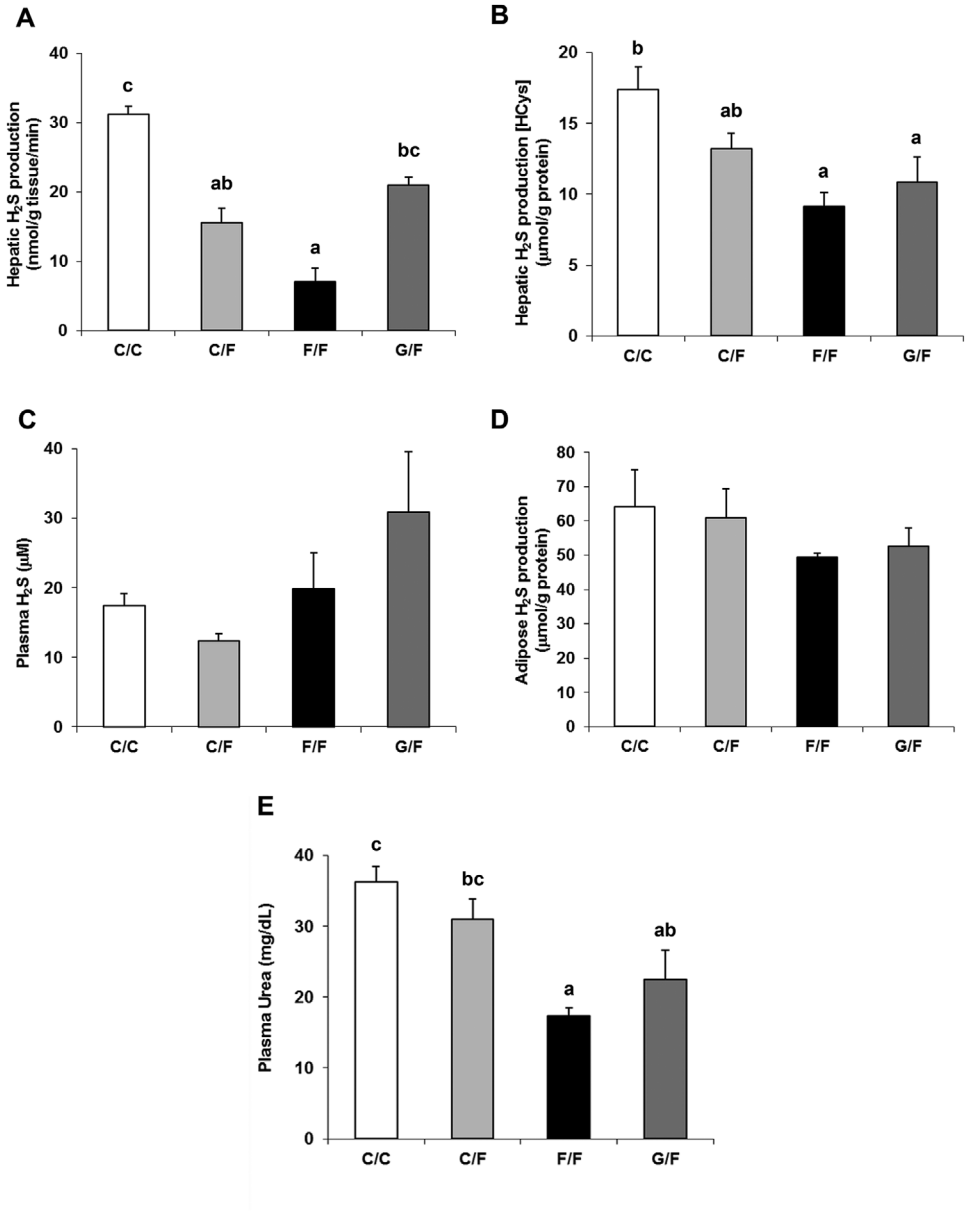


**Figure 4.** Liquid fructose in gestation exacerbates fructose-induced repression of hepatic $H_2S$ production in adult female progeny. A) Hepatic $H_2S$ production from Cys, B) hepatic $H_2S$ production from Hcy, C) plasma $H_2S$ levels, D) adipose tissue $H_2S$ production from Cys, and E) plasma urea levels of fructose-fed female adult progeny from control (C/F, light grey bar), fructose- (F/F, black bar), and glucose-supplemented (G/F, dark grey bar) mothers. C/C: Control female 261-day-old offspring from control pregnant rats (empty bar, C/C). Data are means ± S.E. from 5–6 litters. Values not sharing a common letter are significantly different ($p < 0.05$). Cys: cysteine; Hcys: homocysteine.

and C/F, and almost significant ($p = 0.068$) for G/F rats, versus control (C/C) group. Moreover, when using homocysteine as substrate, the decrease induced by fructose ingestion in hepatic $H_2S$ production was confirmed (Figure 4B), and the differences became significant for F/F and G/F groups versus control (C/C) rats.

However, plasma $H_2S$ was not affected by fructose ingestion (Figure 4C), although a trend to increase was observed in G/F group. Plasma $H_2S$ levels reflect the production of this gas not only from liver, but also from other tissues, such as lung, kidney, and adipose.[21,32] In fact, $H_2S$ generation by lumbar adipose tissue was hardly affected by fructose intake (Figure 4D).

Regarding oxidative stress parameters, fructose-fed rats unexpectedly showed lower hepatic lipid peroxidation (as MDA levels) in comparison to control (C/C) group, becoming significant for the progeny of fructose-fed and glucose-fed mothers (Table S2, Supporting Information). However, neither the hepatic levels of reduced glutathione nor the hepatic SOD2 activity were affected by fructose intake (Table S2, Supporting Information). Curiously, protein oxidation (measured as carbonyls of proteins) displayed an opposite profile to the one observed for lipid peroxidation.

**Table 2.** Molecular regulators of CSE expression and H2S production. Liver gene expression (mRNA) of transcription factors and/or their target genes of 261-day-old fructose-fed female progeny from fructose- (F/F) or glucose-supplemented (G/F) and control mothers (C/F).

| | C/C | C/F | F/F | G/F |
|---|---|---|---|---|
| ATF4 | 1.12 ± 0.19 | 1.08 ± 0.08 | 1.20 ± 0.11 | 1.47 ± 0.12 |
| TNF$\alpha$ | 1.04 ± 0.38 | 0.49 ± 0.09 | 0.42 ± 0.07 | 0.53 ± 0.09 |
| Shp | 4.63 ± 1.00$^{ab)}$ | 3.87 ± 0.62$^{ab)}$ | 4.06 ± 0.63$^{a)}$ | 8.54 ± 1.50$^{b)}$ |
| LRH1 | 11.36 ± 1.30 | 9.51 ± 1.30 | 9.60 ± 0.57 | 12.59 ± 1.21 |
| SLC6A6 | 5.75 ± 0.69$^{b)}$ | 2.11 ± 0.25$^{a)}$ | 2.03 ± 0.41$^{a)}$ | 3.46 ± 0.37$^{a)}$ |
| Calreticulin | 244.8 ± 29.7$^{ab)}$ | 208.0 ± 4.0$^{a)}$ | 286.6 ± 18.9$^{b)}$ | 301.5 ± 19.9$^{b)}$ |
| CHOP | 1.66 ± 0.26$^{ab)}$ | 1.56 ± 0.23$^{a)}$ | 2.23 ± 0.12$^{ab)}$ | 2.67 ± 0.37$^{b)}$ |
| Grp78 | 2.36 ± 0.15$^{a)}$ | 2.29 ± 0.06$^{a)}$ | 3.03 ± 0.21$^{ab)}$ | 3.27 ± 0.27$^{b)}$ |
| Calmodulin 3 | 1.07 ± 0.11$^{ab)}$ | 0.95 ± 0.08$^{a)}$ | 1.34 ± 0.03$^{b)}$ | 1.29 ± 0.07$^{b)}$ |

Data are M ± S.E., from 5–6 litters. Relative target gene mRNA levels were measured by real time PCR, as explained in Section 4, and normalized to Rps29 levels and expressed in arbitrary units (a.u.). Different letters indicate significant differences between the groups ($p < 0.05$). TNF$\alpha$: Tumor necrosis factor alpha; ATF: Activating transcription factor; Shp: Small heterodimer partner; LRH1: Liver receptor homolog 1; SLC: Solute carrier protein; CHOP: C/EBP homologous protein; Grp78: 78 kDa glucose-related protein.

Thus, the three groups of fructose-fed rats showed higher protein oxidation versus the control (C/C) group, becoming significantly different for rats from fructose-fed mothers. In accordance with this, heme oxygenase-1 (HO-1) gene expression, a potent endogenous antioxidant enzyme,[28] was lowered by fructose intake (Table S2, Supporting Information), these effects being significantly different for C/F and G/F, and almost significant ($p = 0.099$) for F/F rats, versus control (C/C) group. Moreover, although the levels of the protein carbonylation marker, methylglyoxal, were not different between the three groups, the concentration of the prooxidant molecule uric acid tended to be augmented in the F/F group (Table S2, Supporting Information), in parallel to the uricemia previously reported in these animals.[5]

Finally, since ammonia ($NH_3$) is produced along with $H_2S$ in the transsulfuration pathway, an interaction between these two gasotransmitters has been proposed.[17] Ammonia produced in transsulfuration participates in the synthesis of urea by the liver[33] and, in accordance with the diminution of hepatic $H_2S$ production found here in fructose-fed rats (Figures 4A,B), plasma urea was also reduced in fructose-fed rats (Figure 4E), becoming significantly different for F/F and G/F groups versus control (C/C) rats. In fact, a significant correlation was observed between plasma urea levels and $H_2S$ production by the liver ($r = 0.617$; $p < 0.006$, using Hcy as substrate; and $r = 0.647$; $P < 0.004$, using Cys as substrate).

Subsequently, we analyzed, from a molecular perspective, the fructose-induced diminution in both hepatic CSE expression and $H_2S$ synthesis and why this effect was more pronounced in F/F females. Thus, we determined mRNA gene expression of transcription factors that regulate CSE transcription directly or indirectly.[27] However, as shown in **Table 2**, either there was no effect (ATF4) or it trended to be similarly reduced, although not significantly, in the three groups receiving fructose (TNF$\alpha$). On the other hand, since the bile-acid-activated farnesoid X receptor (FXR) has been proposed as an activating factor of CSE transcription,[34] and plasma bile acids were affected by fructose intake in our rats in a maternal-intake dependent manner (25.6 ± 3.7; 15.1 ± 3.4; 17.2 ± 2.4; and 33.2 ± 7.3 µM for C/C, C/F, F/F, and G/F, respectively; $p < 0.05$, for C/F vs G/F group, and almost significant, $p = 0.077$ for F/F vs G/F group), several FXR target genes or genes involved in bile acids metabolism were analyzed. Thus, in accordance with the plasma bile acids levels and the mRNA levels of CSE (Figure 3E), the expression of the three genes determined (Shp, LRH1, and SLC6A6) showed similar profiles in response to the fructose intake, with more pronounced reductions for C/F and F/F groups (Table 2). Interestingly, one of the few factors that is able to affect both CSE expression, CSE activity and $H_2S$ production, although with a biphasic effect, is calcium.[27] Therefore, a number of calcium-responding genes were determined. Thus, the expression of the four genes measured (calreticulin, CHOP, Grp78, and calmodulin) displayed similar profiles in response to the fructose intake, with increases for F/F and G/F groups versus the other two groups (Table 2). Remarkably then, fructose intake did modify more clearly CSE expression and activity in females from fructose-fed mothers (F/F) by affecting both FXR transcriptional activity and $Ca^{+2}$-sensing protein expression.

## 3. Discussion

Homocysteine (Hcy) is a non-protein sulfur amino acid normally present in small amounts in the blood. Hyperhomocysteinemia is an independent risk factor for vascular diseases.[35,36] This risk is proportional to the homocysteine concentration,[37] similar to the relationship between plasma cholesterol and coronary heart diseases. The causes of hyperhomocysteinemia are multifactorial including genetics, age and gender, renal function, nutrition, certain diseases, and some drugs.[38] Elevated homocysteine levels can be reduced by consuming folic acid in the form of a dietary supplement,[39] although its protective action on the detrimental effects of hyperhomocysteinemia is unclear.[40]

Hcy plasma levels remain within narrow limits thanks to the two main routes for its elimination. On the one hand, Hcy follows the transulfuration pathway; the first reaction of this route is catalyzed by the enzyme CBS and the second by CSE to give cysteine, $\alpha$-ketobutyrate and ammonia, both of them being PLP-dependent enzymes.[41] On the other hand, the second route of Hcy elimination is its remethylation to methionine, which can follow two different paths: 1) in the liver, the enzyme BHMT remethylates Hcy using betaine as a methyl donor; and 2) in most tissues this remethylation is catalyzed by the enzyme MTR, which uses vitamin $B_{12}$ as a cofactor and 5-methyltetrahydrofolate as a methyl donor.[42]

Curiously, a product of the transsulfuration pathway, $H_2S$ is a gaseous signalling molecule with potential to mediate a wide range of physiological benefits including stress resistance, longevity, vasodilation, neurodegeneration, and atherosclerosis, among others. Moreover, it has been involved in tumor growth.[18,43,44] Since diet can impact $H_2S$ production,[44] it still remains to be established which dietary requirements and nutrients are involved in the control of endogenous $H_2S$ production.

HFD, rich in saturated fats and cholesterol, are known to promote lipid accumulation in the liver, cellular oxidative stress,

tissue inflammation, etc.[16] HFD are also able to modify, although with controversial findings, the transsulfuration pathway in several tissues affecting Hcy plasma levels.[16,21] We and others have reported that diets rich in fructose can also provoke many of the characteristic features of metabolic syndrome such as those above mentioned in HFD fed animals.[5,23,45–47] However, studies on the effects of fructose feeding in the metabolism of Hcy and $H_2S$ are scarce.

In the present work, females born of fructose-fed mothers presented higher homocysteinemia than the offspring from control and glucose-fed dams. This unexpected finding could be due to a decreased capability in female rats from fructose-fed mothers to remethylate Hcy in the liver. And this effect was found using both betaine, since BHMT gene expression was diminished, and folates since, although MTR gene expression was not affected, folic acid plasma levels were diminished. In contrast, the other route for Hcy elimination, the transsulfuration, appeared clearly increased by maternal fructose in female progeny. Both CBS and CSE gene expression were augmented in females from fructose-fed dams. Possibly, an activated transsulfuration pathway could be a compensatory mechanism to ameliorate the high plasma values of Hcy found in this progeny. In accordance with these results, Hcy has been reported to promote the activity of CSE in a concentration-dependent manner[31] although, the opposite effect has also been reported.[48] Moreover, opposite responses of BHMT and CBS have also been previously described supporting our results. Thus, CBS knockout mice showed higher BHMT gene expression than their counterpart wild-type.[49]

In addition to the transsulfuration pathway, CBS and CSE are also responsible for the desulfuration route that generates $H_2S$. In the liver, $H_2S$ production seems to be mainly due to the CSE activity.[31,48] Furthermore, several authors have shown that $H_2S$ could ameliorate the detrimental effects of hyperhomocysteinemia.[18,50] In the present study we demonstrate for the first time that maternal fructose intake provokes an increase in plasma $H_2S$ levels and, more importantly, an activated hepatic production of $H_2S$ in the female offspring, in comparison to the progeny from control and glucose-fed mothers. This augmented $H_2S$ production is in agreement with the increased CSE and CBS gene expression found in the progeny of fructose-fed mothers. Curiously, it seems to be a specific effect for the liver, since different findings were observed in lumbar adipose tissue ($1.47 \pm 0.21$; $1.03 \pm 0.07$; and $0.68 \pm 0.03$ nmol $g^{-1}$ tissue $min^{-1}$ for female offspring from control, fructose- and glucose-fed dams, respectively; $p < 0.05$, for offspring of glucose-fed dams vs progeny of fructose-fed mothers).

Interestingly, in line with the beneficial effects proposed for $H_2S$ against atherosclerosis, NAFLD and other characteristics of metabolic syndrome, $H_2S$ has been inversely related to inflammation, steatosis, and oxidative stress.[16,18,21,48] In the rats of our maternal fructose intake model presented here, liver triglyceride concentration,[4] TNF$\alpha$ expression, (Table 1) and plasma ALT ($66.7 \pm 17.0$; $79.1 \pm 9.2$; $67.0 \pm 7.6$ U $L^{-1}$ for female offspring from control, fructose-, and glucose-fed dams, respectively), parameters related to steatosis and inflammation, respectively, were not affected in the female progeny of fructose-fed mothers. However, hepatic lipid peroxidation was diminished and the activity of manganese superoxide dismutase (SOD), related to the elimination of reactive oxygen species (ROS), was augmented in the progeny of fructose-fed dams. This would be in agreement with previous studies showing that $H_2S$ promotes these effects and alleviates ROS generation.[26,50]

Regulation of CSE/$H_2S$ system is quite complex and many factors are involved.[27] For example, insulin and glucose have been proved to affect CSE expression in cells. However, findings obtained in both insulinemia and glycemia in female offspring from control, fructose-, and glucose-fed dams[4] did not show differences related to the results found here for CSE expression. A similar situation was observed for several nuclear receptors and factors that modulate CSE transcription by targeting transcription factors[27] such as Akt,[4] ATF4, TNF$\alpha$, or calcium (Table 1). CSE gene has also been proposed as a specific target of FXR,[34] although no effects due to maternal diet have been found in other FXR-target genes analyzed in these rats.[19] Therefore, the increase in the hepatic transsulfuration pathway and production of $H_2S$ specifically found here in adult female progeny from fructose-fed mothers could be related to the changes also observed in the Nrf2 and NFAT regulated-pathways. Thus, in order to elucidate the action mechanism, we studied XBP1u and HDAC3 gene expression since it has been demonstrated that unspliced XBP1 and HDAC3 are involved in the formation of a complex that stabilizes Nrf2, and it has been proposed that this transcription factor regulates CSE mRNA expression.[27,29] Since XBP1u expression was clearly augmented in female progeny from fructose-fed dams (Table 1), it could be promoting the formation of the XBP1u/HDAC3 complex which, in turn, would increase Nrf2 stabilization and its transcriptional activity. On the other hand, sites to bind NFAT transcription factors have recently been identified in the CSE promoter.[30] In accordance with this, increases of mRNA levels for several NFAT5 target genes (Table 1), including CSE (Figure 1E), have been found here in females from fructose-supplemented mothers. Nevertheless, in addition to this putative implication of Nrf2 and NFAT in CSE expression for females born to fructose-fed mothers, a role of the maternal diet affecting the methylation status of the CSE promoter should not be discarded, as we have previously reported for CPT1 and LXR genes in these animals.[19]

As well quite interesting and unexpected results were also found in the female progeny submitted to a fructose supplementation when adults. Whereas homocysteinemia tended to be diminished after liquid fructose ingestion in F/F and G/F groups, plasma folic acid levels were markedly reduced after 21 days of fructose intake, independently of whether progeny was born of control, fructose- or glucose-fed mothers. Plasma folate levels are influenced by diet, and we have previously shown that female rats reduced their total amount of energy intake from solid food to almost half (around 52%) by feeding fructose in the drinking water (C/F, F/F, G/F).[5] Therefore, the low amounts of folic acid found here in fructose-supplemented rats would be mostly due to a reduced ingestion of this vitamin in the food.

Possibly, in order to compensate for a reduced remethylation of Hcy from folates (due to a lack of substrate) to generate methionine, the remethylation from betaine through BHMT tended to be increased in the F/F group and the elimination of Hcy by the transsulfuration pathway (through CSE) reduced in fructose-fed rats, mainly in F/F and C/F groups. Again, BHMT and the transsulfuration pathway showed opposite responses. Curiously, CBS was not affected by fructose ingestion except in G/F group,

where it was increased. Peh et al. have also reported, although using a HFD, that CBS can show a different response than CSE to the nutritional challenge,[21] and they attributed this to a compensatory mechanism. This situation was also described in the hearts of mice rendered diabetic after treatment for 15 months with 30% of fructose.[24] However, these authors have related CSE decrease and CBS increase to the hyperglycemia and hyperHcy, respectively, modifications that were also observed in these diabetic mice. In our study, neither hyperhomocysteinemia nor hyperglycemia[5] were found in fructose-fed females.

In accordance with the role of CSE as the principally responsible for $H_2S$ production in liver,[31,48] the hepatic $H_2S$ synthesis dropped due to fructose ingestion in the three experimental groups and, interestingly, it was much more pronounced in the progeny from fructose-fed mothers. Jin et al. (2015), although measuring endogenous $H_2S$ levels instead of production, also found a lesser amount of $H_2S$ in the hearts of ageing diabetic mice and in cardiomyocytes subjected to a high dose of glucose.[24] However, this is the first time that it has been reported that maternal fructose intake influences the response of hepatic $H_2S$ synthesis to high fructose-feeding.

In spite of a clearly reduced production of $H_2S$ in liver, plasma $H_2S$ levels were not affected by fructose ingestion. Other authors, in high-fat fed animals, found that plasma levels of $H_2S$ were not a reflection of the changes observed in the enzyme expression or activity of the liver.[21] In fact, plasma $H_2S$ levels are supposed to be the sum of $H_2S$ production from many tissues: liver, kidney, lung, adipose tissue, etc. However, in contrast to Peh et al., who found a reduction of $H_2S$ production in extrahepatic tissues of high-fat fed animals,[21] we did not find any change in $H_2S$ synthesis in the lumbar adipose tissue of fructose-fed rats.

Several authors have involved an activated endogenous $H_2S$ production (or exogenous administration) with beneficial effects to ameliorate several risks factors of metabolic syndrome, such as lipid deposition, tissue inflammation,[18] and oxidative stress.[16,24,48] In our rats, although inflammation tended to be reduced although not significantly (TNF$\alpha$ gene expression, Table 2, and plasma ALT, 66.7 ± 17.0; 42.5 ± 2.7; 47.4 ± 2.7; and 49.2 ± 4.4 U $L^{-1}$ for C/C, C/F, F/F, and G/F, respectively) and lipid peroxidation (hepatic MDA) was diminished in fructose-fed animals, protein oxidation was augmented and the gene expression of HO-1, a potent antioxidant enzyme, dropped after fructose-feeding (Table S2, Supporting Information). Corroborating our results, hydrogen sulfide has been proven to protect against ROS production in cardiomyocytes through an increased Sirtuin 1 expression.[26] Therefore, the clear reduction in hepatic hydrogen sulfide production observed in animals supplemented with fructose would explain the increased protein oxidation found in these animals. In addition, Sodhi et al. showed that a diminished HO-1, oxidative stress, and a reduced Sirt1 produced by fructose intake induce hepatic lipid deposition.[51] In accordance with this, we have previously reported that liquid fructose throughout gestation exacerbates fructose-induced dyslipidemia, including hepatic steatosis, in adult female progeny.[5] Moreover, it has been suggested that $H_2S$ directly and/or indirectly suppresses ChREBP gene expression,[52] and we have previously described an increased expression of ChREBP, a lipogenic transcription factor, and its target genes in fructose-fed adult females, mainly in the F/F group.[5]

$H_2S$ is a gas molecule produced in the transsulfuration pathway together with another gas with signaling properties, ammonia ($NH_3$).[17] Ammonia from the transsulfuration pathway, along with the one produced from amino acid catabolism, is converted into urea. Several studies have demonstrated that plasma urea is inversely related to hepatic steatosis.[33,53] In the present study, fructose feeding diminished plasma urea and this effect was more pronounced in the F/F females, which also presented lower CSE expression and $H_2S$ production, and higher lipid accumulation in their livers.[5]

Multiple factors could be involved in the effect of fructose ingestion on CSE expression and hepatic $H_2S$ production and the influence of maternal intake that we have observed in the present study. However, some of these factors did not present changes for the three groups receiving fructose in comparison to the control group (C/C), as occurred for ATF4, or they did not show differences in the response to the fructose intake between the three groups, as observed for Akt, insulinemia[5] TNF$\alpha$ (Table 2) or Nrf2 (since HO1 mRNA levels were similarly decreased in all the fructose-fed groups, Table S2, Supporting Information). Further, the different methylation status of the CSE promoter between the three groups receiving fructose, as suggested above, could also be an influencing factor. However, LXR and CPT1 expression (genes where different methylation levels of the promoter between females from control, fructose- and glucose-fed mothers have been already confirmed[19]) did not show differences between the three groups.[5] Interestingly, plasma bile acids levels and liver FXR activity showed similar responses to fructose intake, with more pronounced decreases in the C/F and F/F groups, in accordance with the findings observed for other FXR target gene, CSE.[34] Possibly, a reduced amount of bile acids arriving to the liver in these groups could be maintaining FXR inactivated and, accordingly, its target genes (including CSE) with a lower expression and, therefore, a diminished production of $H_2S$. We do not know the reason for the differences observed in plasma bile acids levels for C/F and F/F versus G/F group, but a possible implication of some component of the enterohepatic cycle should not be discarded, as we have previously suggested.[19] On the other hand, calcium is one of the few factors that can regulate both expression and activity of CSE and, consequently, $H_2S$ production.[27] However, the effects of calcium seem to be biphasic and dependent on both the type of cells and the intracellular concentration of $Ca^{2+}$.[30,54,55] Calcium has been reported to promote the expression/activity of CSE[27] although, the opposite effect has also been reported.[30,54,55] In our model, fructose could be promoting calcium release of the endoplasmic reticulum (ER) either directly or indirectly,[56] and it is known that a decrease in [$Ca^{2+}$] ER activates the ER stress response increasing, among others, chaperone production such as calreticulin, CHOP, and Grp78.[57,58] Further, an increase in the cytoplasmic calcium concentration would affect $Ca^{2+}$-sensing proteins such as calmodulin.[59] Thus, increases in the gene expression of all these calcium related proteins were observed in F/F and G/F groups, but not in C/F group (Table 2). These results would indicate that calcium is exerting a negative effect on both CSE expression and activity in the F/F group in accordance to Mikami et al.,[54] and although calcium is not able to affect CSE expression in the G/F group, it effectively modifies CSE activity. In any case, it is remarkable that fructose intake was able to affect both bile acids,

FXR activity, and calcium-related protein expression exclusively in females from fructose-fed mothers (F/F) and, possibly, this was the reason for finding a more pronounced fructose-induced diminution on CSE expression and activity in this group.

In summary, we have demonstrated that maternal fructose intake promotes the hepatic production of $H_2S$, a gasotransmitter with proved benefits in atherosclerosis and metabolic syndrome, in adult female offspring. However, one of the most prominent results found here is that maternal fructose intake exacerbates the fructose-induced fall in the hepatic synthesis of $H_2S$ in adult female progeny. The possible connection between this profound decrease in the production of this beneficial gas, liver steatosis, and oxidative stress, suggests to us a reduction in the consumption of fructose-sweetened beverages, especially during gestation, around all the world. Furthermore, it is quite remarkable that, as we and others have demonstrated,[28,51,60] and the present study, fructose intake negatively acts against systems involved in the endogenous production of NO, CO, $H_2S$, and $NH_3$, all of them being molecules recognized as gasotransmitters.[17] Thus, bearing in mind the potential benefits of these gases in ameliorating many common non-communicable diseases such as atherosclerosis, metabolic syndrome, etc., we would like to reinforce the notable clinical relevance of the observations obtained in the present study.

## 4. Experimental Section

*Animals and Experimental Design*: An animal model of maternal liquid fructose intake was developed as previously described.[4,7,19] Female Sprague-Dawley rats weighing 200–240 g were fed ad libitum, a standard rat chow diet (B&K Universal, Barcelona, Spain), and housed under controlled light and temperature conditions (12 h light-dark cycle; 22 ± 1 °C). The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU, Madrid, Spain (ref. number 10/206 458.9/13). Pregnant animals were randomly separated into a control group, a fructose-supplemented group (fructose), and a glucose-supplemented group (glucose) (5–6 rats per group). Fructose and glucose were supplied as a 10% (wt/vol) solution in drinking water throughout gestation. Control animals received no supplementary sugar. Pregnant rats were allowed to deliver and on the day of birth, each suckling litter was reduced to 9 pups per mother. After delivery, both mothers and their pups were maintained with water and food ad libitum. At 21 days of age, pups were separated by gender and kept feeding on a standard rat chow diet (B&K Universal, Barcelona, Spain) and water without additives. Animals within each experimental group were born to different dams to minimize the "litter effect."

At 240 days of age, one half of the female descendants were randomly separated. When the progeny was 261-day-old, it was sacrificed and blood and tissues were collected. Remarkably, these animals had received no subsequent additive in the drinking water for their entire lives.

The other half of the female progeny was subjected to the following protocol: First, they were weighed and an aliquot of plasma was obtained from the tail in order to confirm that the values between the experimental groups both for body weight and for several analytes (glucose, triglycerides, non-esterified fatty acids, etc.) were similar.[5] Then, vaginal smears were carried out to confirm that all the female rats were at the same period of the cycle. Later, independently from the experimental group of mothers to which they had been born, they were maintained on solid pellets and supplied with drinking water containing 10% (wt/vol) fructose. Thus, three experimental groups were formed: C/F, F/F, G/F, the first letter indicating whether the mothers had been supplied during pregnancy with tap water (C, control), or water containing a carbohydrate (F: fructose; G: glucose); and the second letter indicating the period with fructose (F), when they were adults. When the descendants were 261-days-old, they were decapitated at 10 a.m. and blood was collected using tubes containing $Na_2$-EDTA. Prior to sacrifice, food was removed at 8 a.m. The period with fructose was selected to last 21 days (from 240 to 261 days of age) as previously reported.[5,45] Liver and adipose tissue were immediately removed, placed in liquid nitrogen and kept at −80 °C until analysis. Samples were then centrifuged, and plasma was stored at −80 °C until processed for later determinations. In parallel, a fourth experimental group was used, C/C: female progeny from control mothers supplied with water without additives when adult.[5,45] Plasma 17$\beta$-estradiol concentrations were similar between the four groups, indicating that all the female rats had been sacrificed at the same period of the cycle.[5] The males of each litter were used for a separate experiment.[4,45]

*Determinations*: Plasma aliquots were used to measure homocysteine and urea by enzymatic colorimetric tests using commercial kits (Spinreact, Girona, Spain). An enzymatic kit using chemiluminescent immunoanalysis on microparticles (Architect, Abbot, Ireland) was used for the quantitative determination of folic acid in plasma.

Plasma $H_2S$ levels were measured using the methylene blue method as previously described with some modifications.[61] Briefly, 200 µL of plasma were deproteinized with 150 µL 20% TCA. After centrifugation, 250 µL of supernatant were treated with 150 µL 1% zinc acetate, 100 µL 20 mM N1,N1- dimethylbenzene-1.4-diamine sulfate (Fluorochem, UK) in 7.2 M HCl and 133 µL 30 mM $FeCl_3$ (Panreac AppliChem, IL, USA) in 1.2 M HCl. Then, samples were vortexed and incubated at room temperature for 20 min and absorbance measured at 630 nm. A standard curve from 1.56 to 200 µM of sodium hydrosulfide (NaHS, Fluorochem, UK) was performed following the same procedure as with the samples.

*Total RNA Preparation and Analysis*: Total RNA was isolated from liver using Ribopure (ThermoFisher Scientific, USA). Total RNA was subjected to DNase I treatment using Turbo DNA-free (Invitrogen, ThermoFisher Scientific, USA), and RNA integrity was confirmed by agarose gel electrophoresis. Afterwards, cDNA was synthesized by oligo(dT)-primed reverse transcription with Superscript II (Invitrogen, ThermoFisher Scientific, USA). qPCRs were performed using a Light Cycler 1.5 (Roche, Germany). The reaction solution was carried out in a volume of 20 µL, containing 10 pmol of both forward and reverse primers, 10x SYBR Premix Ex Taq (Takara Bio Inc., Japan) and the appropriate nanograms of the cDNA stock. Rps29 was used as a reference gene for qPCR. The primer sequences were obtained either from the Atlas RT-PCR Primer Sequences (Clontech, CA, USA) or designed using Primer3 software (University of Massachusetts Medical School, MA, USA).[62] Samples were analyzed in duplicate on each assay. Amplification of non-specific targets was discarded using the melting curve analysis method for each amplicon. qPCR efficiency and linearity were assessed by optimization of the standard curves for each target. The transcription was quantified with Light Cycler Software 4.05 (Roche, Germany) using the efficiency correction method.[63]

*Determination of $H_2S$ Production by Liver and Adipose Tissue*: $H_2S$ production in liver and lumbar adipose tissue was evaluated following the lead sulfide method as previously described with modifications.[64] Briefly, liver and adipose tissue were homogenized in phosphate-buffered saline and their protein levels measured with the BCA Assay Kit (Thermo Fisher, MA, USA). 100–300 µg of protein were incubated at 37 °C in the presence of 10 mM Cys (Sigma-Aldrich, MO, USA) or 30 mM Hcy (Fluorochem, UK), and 20 µM Pyridoxal 5′-phosphate (PLP) (Sigma-Aldrich, MO, USA) (for livers) or 2 mM PLP (for adipose tissue) on 96 well plates covered with a lead acetate (II) membrane. Incubations were performed until dots of lead sulfide were detected but not saturated, and this occurred after 2 h for liver and 6 h for fat pads. In order to prepare these membranes, Whatman n° 2 paper was soaked into 20 mM lead acetate (Sigma-Aldrich, MO, USA) and vacuum dried. Dots were densitometered (BioRad Densitometer G-800, CA, USA) for quantification. A standard curve from 0 to 1000 mM NaHS was performed for each membrane.

*Determination of Oxidative Stress Parameters*: 100 mg of frozen liver were homogenized into 0.25 M Tris-HCl, 0.2 M sucrose, and 5 mM dithiothreitol pH 7.4 buffer. The concentration of malondialdehyde (MDA) was measured as a marker of lipid peroxidation using the method previously described,[65] by assessing the fluorescence of MDA-thiobarbituric acid

complexes at 515 nm/553 nm excitation/emission wavelengths. As a protein oxidative stress biomarker, protein carbonyls content was determined using the method previously described.[66]

Tissue samples were also used to evaluate the concentration of reduced glutathione (GSH), using capillary electrophoresis coupled to UV-DAD and following the method previously reported.[67] Finally, the activity of the enzyme SOD was measured using a commercial kit (Sigma-Aldrich, MO, USA).

*Statistical Analysis*: Results were expressed as means ± S.E. Treatment effects were analyzed by one-way analysis of variance. When treatment effects were significantly different ($p < 0.05$), means were tested by Tukey´s multiple range test, using the computer program SPSS (version 23). When the variance was not homogeneous, a post hoc Tamhane test was performed.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

C.B. conceived and designed the study. E.F., S.R., R.A., C.D., P.O., and M.I.P. contributed reagents/materials/analysis tools for gene expression studies and parameter analysis. M.I.P., P.O., and S.R. carried out the analysis for oxidative stress studies. L.R. handled the animals. M.I.P. and J.J.A.-M. analyzed the data. C.B. wrote the paper.

## Keywords

fetal programming, fructose, hydrogen sulfide, liver, oxidative stress

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