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Maternal high-fat intake during pregnancy programs metabolic-syndrome-related phenotypes through liver mitochondrial DNA copy number and transcriptional activity of liver *PPARGC1A*☆,☆☆

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Abstract

In this study, we contrasted the hypothesis that maternal diet during pregnancy has an impact on fetal metabolic programming through changes in liver mitochondrial DNA (mtDNA) content and transcriptional activity of *Ppargc1a* and that these effects are sex specific.
Methods: Rats were fed either high-fat (HFD) or standard chow diet (SCD) during gestation and lactation. The resulting adult male and female offspring were fed either HFD or SCD for an 18-week period, generating eight experimental groups.
Results: Maternal HFD feeding during pregnancy is associated with a decreased liver mtDNA copy number ($P<.008$). This effect was independent of the offspring sex or diet, and was significantly associated with fatty liver when dams were fed HFD ($P<.05$, adjusted by homeostasis model assessment of insulin resistance, HOMA-IR). We also found that maternal HFD feeding results in a male-specific significant reduction of the liver abundance of *Ppargc1a* mRNA ($P<.004$), which significantly impacted peripheral insulin resistance. Liver expression of *Ppargc1a* was inversely correlated with HOMA-IR ($R=-0.53$, $P<.0003$). Only male offspring exposed to a chronic metabolic insult in adult life were insulin resistant and hyperleptinemic, and showed abnormal liver and abdominal fat accumulation. Liver abundance of *Tfam*, *Nrf1*, *Hnf4a*, *Pepck* and *Ppparg* mRNA was not associated with maternal programming. In conclusion, maternal high-fat diet feeding during pregnancy programs liver mtDNA content and the transcriptional activity of *Ppargc1a*, which strongly modulates, in a sex-specific manner, glucose homeostasis and organ fat accumulation in adult life after exposure to a nutritional insult.
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Keywords: Metabolic programming; Mitochondrial DNA; *PGC1a*; *PPARGC1a*; Mitochondrial copy number; NAFLD; High-fat diet; Insulin resistance; Liver; Insulin resistance; Gene expression

1. Introduction

The worldwide prevalence of metabolic syndrome (MS) is alarming, having reached pandemic proportions not only in the adult population [1] but also in children [2]. Many factors are involved in this expanded clinical disorder, including the complex interaction between a genetic predisposing background and the exposure to an environment of overnutrition characterized by diets rich in fat and carbohydrates [3,4]. There is evidence that the close interaction between genes and the environment starts very early in life and can be passed across generations. Because of this phenomenon, the concept of fetal metabolic programming has been proposed, as recently reviewed [5]. This concept emerged after epidemiological observations showed that MS-related diseases, including cardiovascular disease, obesity and diabetes, may be consequences of the 'programming' of the body's structure, physiology and metabolism by the environment during fetal life [6]. Actually, these observations inspired the fetal origin hypothesis of adult diseases, which assumes

Abbreviations: *Actb*, beta-actin; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; HOMA-IR, homeostasis model assessment of insulin resistance; mtDNA, mitochondrial DNA; MS, metabolic syndrome; nDNA, nuclear DNA; *Hnf4a*, hepatocyte nuclear factor 4, alpha gene; *Nrf1*, nuclear respiratory factor-1 gene; *PPARGC1A*/*Ppargc1a*, human/ rat peroxisome proliferator-activated receptor gamma coactivator-1 alpha gene; *Pepck*, phosphoenolpyruvate carboxykinase 1 gene; *Pparg*, peroxisome proliferator-activated receptor gamma gene; *Ppia*, peptidyl prolyl isomerase A (cyclophilin A) gene; *Tfam*, mitochondrial transcription factor A gene.

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that fetal undernutrition in middle to late gestation not only leads to disproportionate fetal growth but also programs long-term changes in physiology and metabolism [7]. Interestingly, recent evidence from animal and human studies has shown that the implications of early nutrition programming are not limited to fetal undernutrition. On the contrary, the increased prevalence of obesity-related diseases observed in the last decades supports a critical role of the opposite fetal scenario characterized by overnutrition. In fact, animal studies have shown that rats that underwent either a high-carbohydrate [8] or a high-fat (HFD) [9] dietary modification during pregnancy are able to transmit the MS-related phenotypes to their offspring, thus establishing a generational effect.

Programming studies mostly focus on changes in target gene expression that modify the phenotype in the progeny. Recent evidence from experimental animals has shown that mitochondrial dysfunction may be a long-term consequence of a poor nutritional environment during early life [10–12]; similarly, exposure to a maternal diet rich in animal fat is associated with altered mitochondrial gene expression [13]. These studies opened the question on the putative role of nutritional mitochondrial programming. We have reported that both extremes of neonatal birth weight are associated with decreased umbilical cord mitochondrial DNA (mtDNA) content [14]. Furthermore, in a recently published human study that included patients with all features of MS, including nonalcoholic fatty liver disease (NAFLD, the hepatic manifestation of the MS), we observed that both the liver transcriptional activity of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (*PPARGC1A*) and a change in the liver mtDNA content were associated with peripheral insulin resistance [15]. Hence, we supported the role of the fatty liver as a strong modifier of and/or a principal player in the natural history of MS-related diseases.

In view of all the evidence described and considering the strong influence of the maternal environment during fetal life on modeling the metabolic profile of the progeny, we hypothesized that maternal high-fat feeding during pregnancy is associated with a programming effect on the liver abundance of *Ppargc1a* mRNA, predisposing the offspring to develop insulin resistance and MS-related phenotypes when they are exposed to a metabolic insult in later life. In addition, we hypothesized that maternal programming modulates liver mtDNA content.

To address these hypotheses, we measured the liver mtDNA copy number in the adult offspring of dams fed HFD during gestation and lactation. We additionally examined the liver mRNA abundance of nuclear genes involved in the regulation of mtDNA transcription and replication, including *Ppargc1a*, also known as *PGC1α*, a master transcriptional coactivator involved in the regulation of mitochondrial biogenesis. Finally, we also aimed to test whether the effect on fetal programming was sex specific, as we had found preliminary evidence that this may be the case [16].

2. Materials and methods

2.1. Animals and study design

Twelve-week-old female Wistar rats of first-order parity and weighing 228±10 g were randomly assigned to either *ad libitum* HFD solid diet (40% wt/wt bovine and porcine fat added to the standard chow) [17] or standard chow diet (SCD). The HFD provided 5340–5460 (kcal/kg) vs. the SCD's 2900–3100 (kcal/kg), and 13.8% of proteins vs. 23%, respectively. The amount of proteins in the HFD was adequate as recommended by nutrition guidelines for rats to be maintained after a rapid growth [18].

Dams were fed 15 days before conception and during gestation and lactation; weight gain and food intake were measured at these periods. Upon birth, pups were sexed, and litter size was noted. To ensure homogeneity of offspring evaluated, within 24 h of birth, all litters studied were adjusted to 9/10 pups per dam; the sex ratio was maintained as close to 1:1 as possible in order to study potential sexual dimorphism. Thus, each experimental group has offspring of two or three mothers; pups continued to be weighed every week.

The offspring at the age of 17 weeks were randomly assigned either *ad libitum* HFD or SCD for an 18-week period, generating eight experimental groups according to male (M) or female (F) sex distribution and maternal HFD or SCD feeding.

All the animals were housed individually, with food and water freely available, and were maintained at room temperature (23°C+1°C) under a 12-h light/dark cycle.

At the completion of the study, the animals were sacrificed by decapitation, and blood samples were collected in tubes containing sodium EDTA and centrifuged; plasma was immediately frozen.

Food was withdrawn from 8:00 a.m. to 4:00 p.m. before the rats were sacrificed, and the intraperitoneal and retroperitoneal fats were measured by weighting them directly.

The liver was quickly snap-frozen and stored at −76°C until gene expression analysis. A portion of each liver was fixed in 10% formalin for histological analysis.

All the animals received humane care, and the studies were conducted according to the regulations for the use and care of experimental animals.

2.2. Biochemical measurements

Serum and sodium EDTA-plasma were obtained by centrifugation and stored at −80°C until needed. Fasting glucose was measured by an automatic biochemical analytical system (Architect, Abbott, Buenos Aires, Argentina). The plasma insulin levels were determined using a commercial quantitative ultrasensitive enzyme-linked immunosorbent assay rat kit according to the manufacturer's instructions (Crystal Chem Inc., Downers Grove, IL, USA). Insulin resistance was calculated using the homeostasis model of assessment (HOMA-IR) index [fasting plasma insulin (μU/ml)×fasting plasma glucose (mmol/L)/22.5].

2.3. Histological analysis of liver tissue

Formalin-fixed liver tissue was processed, and 5-μm-thick paraffin sections were stained with hematoxylin and eosin (H&E). The degree of steatosis was assessed irrespective of the experimental groups as previously described [19] based on the percentage of hepatocytes containing macrovesicular fat droplets: grade 0, no steatosis or steatosis <5%; grade 1, >5% to <33% of hepatocytes containing macrovesicular fat droplets; grade 2, >33% to 66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets.

2.4. Quantification of mtDNA

We used an assay based on real-time quantitative polymerase chain reaction (PCR) for both nDNA and mtDNA quantification, with SYBR-Green as a fluorescent dye (Invitrogen, Buenos Aires, Argentina). For the detection of liver nDNA, we selected *GAPDH* between the nucleotides 1443 and 1571, and for the detection of mtDNA, we selected the mitochondrial-encoded 16S RNA (*Rnr2*) between the nucleotides 2451 and 2583 [20]. The results were presented as the mitochondrial DNA/nuclear DNA ratio (mtDNA/nDNA). Duplicate amplifications of mitochondrial and nuclear products were performed separately.

Real-time quantitative PCR was carried out in a BioRad iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The calculation of DNA copy number involved extrapolation from the fluorescence readings in the mode of background subtracted from the readings of the BioRad iCycler according to the above-described procedure for gene expression. Melting curve analysis was carried out at the end of each run to confirm the specificity of amplification and the absence of primer dimers. The two target amplicon sequences (mtDNA and nDNA) were visualized in 2% agarose and purified using the Qiagen Qiaex II Gel Extraction Kit (Tecnolab, Buenos Aires, Argentina). The dilutions of the purified amplicons were used as the standard curve to verify the PCR-efficiency values estimated by using the procedure described below.

2.5. RNA preparation and real-time reverse transcriptase (RT)-PCR for quantitative assessment of mRNA expression

Total RNA was prepared from rat livers using the phenol extraction step method, with an additional DNAse digestion step. For RT-PCR, 3 μg of total RNA was reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega, WI, USA). Real-time PCR was performed for quantitative assessment of mRNA expression in an iCycler thermocycler (BioRad, Hercules, CA, USA) using the fluorescent dye SYBR-Green (Invitrogen, Buenos Aires, Argentina). All the real-time PCRs were run in duplicate, and all the samples of the experimental groups were tested. The relative expression of the target genes' mRNA was normalized to the amount of a housekeeping gene (peptidyl prolyl isomerase A, Ppia, also termed as cyclophilin A) to carry out comparisons between the groups. Cyclophilin was found to be the most stable reference gene for testing liver mRNA expression among other housekeeping genes tested before starting the experiment [β-actin, TATA box binding protein, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh)]. The levels of mRNA were expressed as the ratio of the estimated amount of the target gene relative to the Ppia mRNA levels using fluorescence threshold cycle values calculated for each sample, and the estimated efficiency of the PCR for each product was expressed as the average of each sample efficiency value obtained [21].

The specificity of amplification and the absence of primer dimers were confirmed using melting curve analysis at the end of each run. Supplementary material shows the primer sequences and the resulting PCR product lengths.

2.6. Statistical analysis

Quantitative data were expressed as mean±S.E. As most of the variables were ratios and not normally distributed, and nonhomogeneous variances between the groups were evident, they were compared by the nonparametric Mann–Whitney test or were log-transformed for pairwise mean comparisons by Newman–Keuls test after analysis of variance (ANOVA) using dam diet, offspring sex or offspring diet as categorical variables or analysis of covariance (ANCOVA) for adjusting by cofactors. For instance, data were also adjusted for HOMA-IR whenever applicable. To test the differences in steatosis gradation (as a categorical response variable), we used a model with ordinal multinomial distribution and probit as a link function, with animal length and adipose tissue as continuous predictor variables. The correlation between two variables was determined using Pearson correlation test on log-transformed variables. A value of $P<.05$ was considered statistically significant. We used the Statistica program package StatSoft (Tulsa, OK, USA) to perform all the analyses.

3. Experimental results

3.1. Dam body weight and food intake analysis

Female rats were fed an HFD or SCD diet for 2 weeks before breeding and continued on this diet throughout gestation and lactation. Body weight was measured weekly before conception and during gestation. Baseline body weight was not significantly different between groups (199.3±3.4 g vs. 200.7±3.4 g, P=.9, HFD and SCD, respectively), and by 2 weeks on the diet, the difference in body weight was still nonsignificant between groups (221.4±3.6 g vs. 213.8±3.6 g, P=.63, HFD and SCD, respectively). By the end of gestation, despite a trend toward body weight gain, maternal body weight in the HFD (265.7± 8.3 g) group did not differ significantly from that in control animals (241.3±8.4 g) (P=.41).

During the whole experimental period, HFD dams had a small but significant decrease in the average daily food intake (12.8±1.0 g/day) compared with the control group (15.9±0.9 g/day) ($P<.05$).

3.2. Maternal high-fat feeding during pregnancy programs liver mtDNA copy number

The liver mtDNA content in the offspring was significantly ($P<.008$) reduced when dams were fed HFD, regardless of the offspring sex and diet (Fig. 1A); nevertheless, there was a trend toward a lower liver mtDNA content when offspring were fed HFD ($P<.07$), (Fig. 1B). In addition, offspring liver mtDNA copy number was significantly associated with fatty liver when dams were fed HFD ($P<.05$, adjusted by HOMA-IR).

3.3. Liver abundance of Ppargc1a mRNA is associated with maternal programming and modulates peripheral insulin resistance

To evaluate whether the hepatic gene expression of *Ppargc1a*, a key transcription factor that influences mitochondrial biogenesis, was associated with metabolic programming, we measured its mRNA abundance in the offspring of all experimental groups. We observed that liver *Ppargc1a* mRNA was significantly associated with offspring sex and that it significantly interacts with maternal HFD feeding ($P<.004$), being significantly lower in male offspring of dams fed HFD in comparison with male offspring of dams fed SCD and female offspring of dams fed HFD (Fig. 2). Surprisingly, female offspring of dams fed HFD showed significantly higher liver abundance of *Ppargc1a* mRNA when compared with female offspring of dams fed SCD (Fig. 2).

The liver expression of *Ppargc1a* was inversely correlated with indices of peripheral insulin resistance as measured by HOMA-IR (R=−0.53, $P<.0003$, Fig. 3).

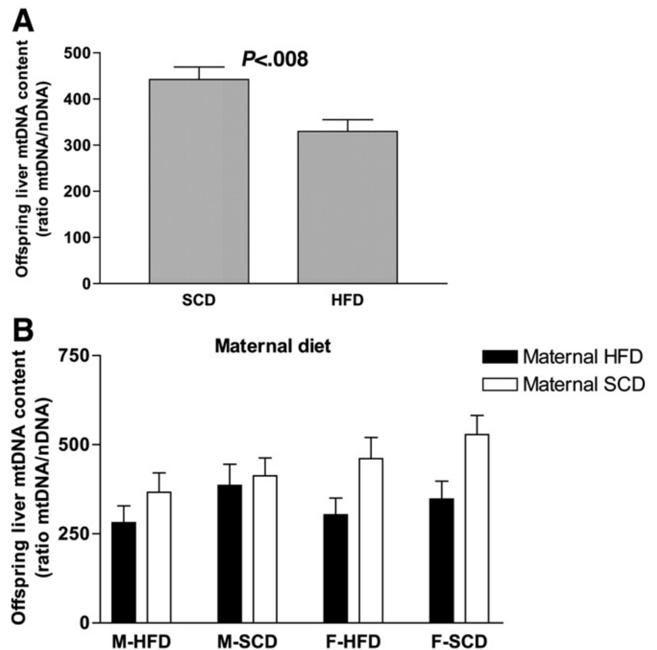

Fig. 1. Programming effect of maternal high-fat diet feeding during pregnancy on liver mtDNA copy number. (A) Liver mtDNA content in the offspring (measured as described in B) was significantly reduced when dams were fed with HDF, regardless of the offspring sex and diet in adult life. (B) Results according to the eight experimental groups. Each bar represents the mean ± S.E. values (nine observations per group) of mtDNA/nDNA ratio assessed by quantitative real-time PCR in each experimental group. M: male, F: female. mtDNA copy number was calculated as the ratio of the mitochondrial 16S RNA gene to the nuclear *Gapdh* gene copy number (ratio mtDNA/nDNA).

Because transcription of the mitochondrial genome is co-regulated with other nuclear genes in addition to *Ppargc1a*, we also explored the effect of maternal programming on the liver abundance of mitochondrial transcription factor (*Tfam,* a factor required for the replication and maintenance of mtDNA), nuclear respiratory factor-1 (*Nrf1*, a gene involved in oxidative-stress-induced mitochondrial biogenesis) and liver peroxisome proliferator-activated receptor gamma (*Pparg*, a regulator of adipocyte differentiation, insulin sensitivity and control of glucose homeostasis) mRNA. We observed that the mRNA expression of the above-mentioned genes in the liver was not associated with maternal programming (Supplementary Figure 1).

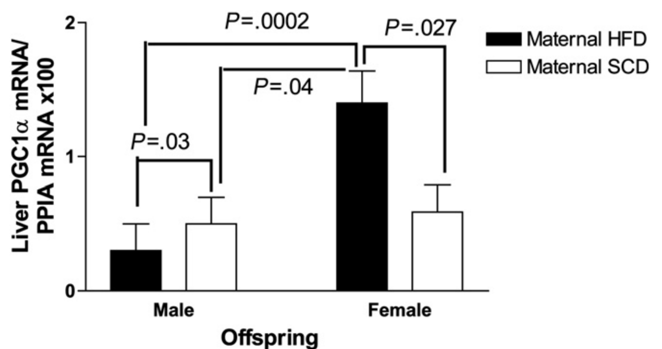

Fig. 2. Maternal high-fat intake during pregnancy programs liver transcriptional activity of *Ppargc1a* (*PGC1α*) in offspring. Liver mRNA expression of *PGC1α* was assessed by quantitative real-time PCR in each experimental group. Each bar represents the mean±S.E. per group according to maternal diet and offspring sex. In each sample, the gene expression was normalized to the expression of cyclophilin A (Ppia) and multiplied by 100.

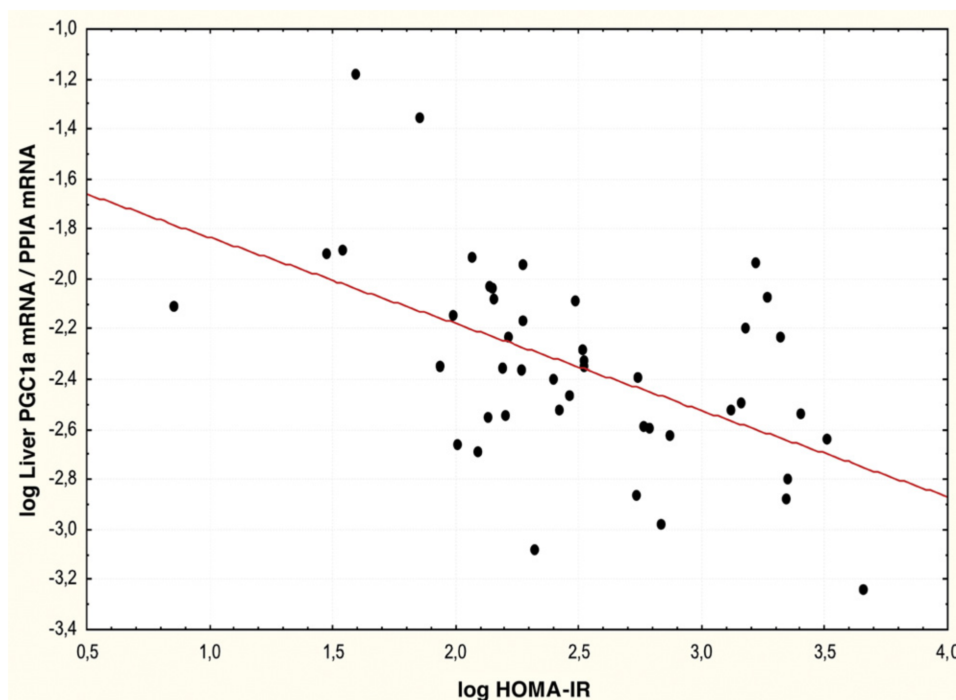

Fig. 3. Peripheral insulin resistance is associated with decreased liver expression of *PGC1α* mRNA. Picture shows an inverse correlation between the log-transformed *PGC1α* mRNA/ cyclophilin A (Ppia) mRNA ratio and the log-transformed HOMA-IR index evaluated in all the animals.

Nevertheless, we observed that the hepatic expression of *Nrf1* and *Pparg* mRNA positively correlated with the liver mtDNA copy number (R=0.62, P<.003 and R=0.51, P<.02, respectively).

3.4. Male-specific programming effect of maternal HFD feeding on insulin resistance

We observed that HOMA-IR was significantly higher in male offspring in comparison with females when dams were fed HFD (P<.04). Interestingly, the effect of programming on insulin resistance was regardless of the exposure to a chronic metabolic insult in adult life, as male offspring showed a significantly higher HOMA-IR index independently of the diet when their mothers were fed with HFD during late pregnancy (Fig. 4). In contrast, maternal HFD feeding did not alter the status of insulin resistance in female offspring; this effect of maternal feeding on HOMA-IR in female offspring parallels the behavior of liver *Ppargc1a* mRNA expression observed in the same group, as female offspring of dams fed HFD showed significantly higher liver expression of *Ppargc1a* mRNA in comparison with female offspring of dams fed SCD (Fig. 2).

Finally, as expected, exposure of offspring to long-term HFD was significantly associated with insulin resistance, regardless of the sex (P<.004). Nevertheless, the HOMA-IR index was significantly higher in male offspring in comparison with females (P<.002, Fig. 4).

To further evaluate the liver transcriptional profile of *Ppargc1a* target genes involved in glucose metabolism, we measured the liver mRNA expression of the hepatocyte nuclear factor 4, alpha gene (*Hnf4α*), a critical component of *Ppargc1a*-mediated gluconeogenesis cascade [22], and the liver mRNA abundance of phosphoenolpyruvate carboxykinase 1 gene (*Pepck*), which is critically involved in the rate-controlling step of the gluconeogenic pathway and is supposed to be modulated by the *Ppargc1a* transcriptional activity.

We observed that liver mRNA expression of *Hnf4a* was not associated either with maternal or offspring diet, or offspring sex (Supplementary Figure 2). Interestingly, we observed that long-term feeding of offspring with HFD was significantly associated with a down-regulation of the liver *Pepck* mRNA (P<.02), regardless of the offspring sex. In addition, we found that the liver abundance of *Pepck* mRNA was significantly lower in female offspring (P<.001); these data support our results showing that females have better adaptation to metabolic demands when compared with males, as they are able to modulate glucose metabolism by inhibition of glucose production from hepatic gluconeogenesis (Supplementary Figure 2). Nevertheless, the observed effect of HFD on the liver *Pepck* mRNA expression in offspring was not significantly related to the maternal diet, although a trend toward a difference was observed when adjusted by HOMA index (P<.07). Thus, we may speculate that this effect is not associated with maternal programming, but more experiments are needed to follow up this result.

3.5. MS-related phenotypes of offspring chronically exposed to a nutritional insult is under programming effect and shows sexual dimorphism

Previous evidence had shown that the programming effect on some phenotypes associated with the MS, such as cardiovascular dysfunction, is sex specific [23–25]. To further evaluate whether HFD prenatal programming also shows a sexually dimorphic effect on MS-related phenotypes, including fatty liver disease, we compared male and female offspring to contrast the hypothesis that females whose mothers had consumed HFD might be protected from organ fat accumulation when exposed to a nutritional insult in adult life.

We observed that after chronic exposure to a long-term metabolic insult (HFD), males whose mothers had consumed HFD had increased abdominal fat content and serum leptin levels in comparison with those whose mothers had consumed SCD (Fig. 5). Interestingly, this effect was not observed in female offspring under the same experimental conditions (Fig. 5), suggesting a sex-specific programming effect on MS-related phenotypes. Likewise, among male offspring, maternal HFD feeding during pregnancy was significantly

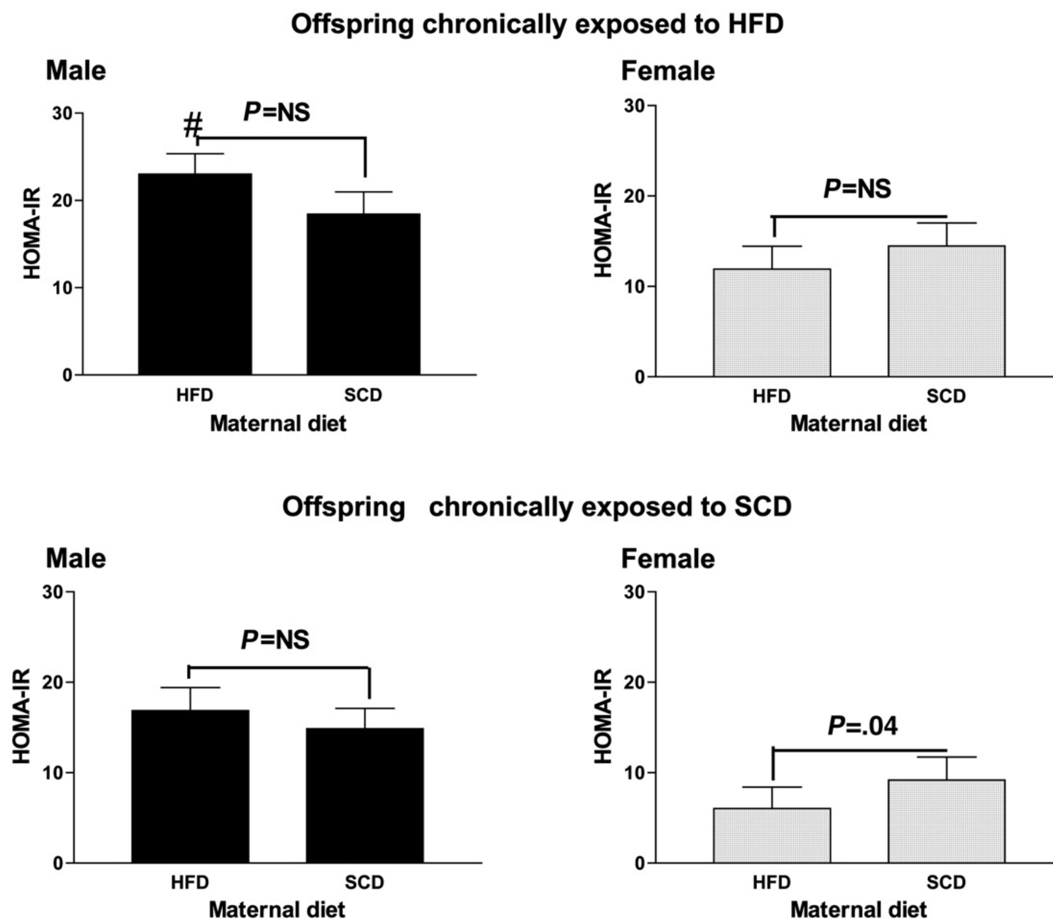

Fig. 4. Offspring of dams fed with a high-fat diet exhibit a sex-dependent enhancement of the insulin resistance index (HOMA-IR). Each bar represents the mean±S.E. for nine observations per group. #P<.01 male offspring–HFD-fed dam vs. female offspring–HFD-fed dam. Statistical significance was defined as P<.05. P values in each panel refer to comparison between offspring of HFD vs. SCD dam groups for each offspring sex and diet in the adult life.

associated with higher scores of fatty liver disease (Fig. 6). On the contrary, not only did maternal HFD feeding not alter the susceptibility of female offspring to fatty liver disease after exposure to a nutritional insult, but it also seems to have a protective role.

4. Discussion

In this study, we contrasted the hypothesis that maternal diet intervention during pregnancy has an impact on metabolic programming by modifying liver mtDNA content and the transcriptional activity of *Ppargc1a*. We suggested that both factors might influence the susceptibility to develop MS-related phenotypes in later life after exposure to a nutritional insult. Furthermore, we hypothesized that the metabolic programming effect is sex specific.

Interestingly, we observed that maternal HFD feeding during pregnancy is associated with decreased liver mtDNA copy number; this effect was not influenced either by offspring sex or by diet in adult life. In addition, liver mtDNA was significantly associated with fatty liver when dams were fed with HFD, and showed a significant interaction with the sex of the offspring. We additionally found that maternal HFD results in a male-specific significant reduction in hepatic *Ppargc1a* mRNA abundance, and this reduction is significantly associated with peripheral insulin resistance in male offspring. In line with this result, we observed that offspring exposed to a chronic metabolic insult in adult life behave differently when they are male and born of HFD-fed mothers. They were insulin resistant and hyperleptinemic, and showed abnormal liver and abdominal fat accumulation. Conversely, we observed that not only did maternal fat intake not cause MS in female offspring, but such offspring also seemed to be protected from programmed insulin resistance and organ fat accumulation, including fatty liver disease. Surprisingly, this observation was consistent with the increased transcriptional activity of liver *Ppargc1a*, which was significantly higher in females born of HFD-fed mothers in comparison with males in the same experimental condition and even with female offspring born of SCD-fed mothers.

Taken together, our results strongly support the hypothesis on the critical role of “liver programming” on the development of insulin resistance and MS in adult life. This programming seems to implicate two critical molecular targets: mtDNA and a master transcriptional coactivator, *Ppargc1a*.

Regarding the observed changes in liver mtDNA, previous experimental studies had demonstrated that programming of mitochondrial dysfunction is a consequence of maternal malnutrition [12]. Nevertheless, we add some novel clues on a putative underlying mechanism that may explain why mitochondria do not function properly in active tissues under metabolic stress, such as the liver, owing to a decrease in the number of copies of mtDNA (mammalian mitochondrial genome encodes 13 of the proteins present in the respiratory chain complexes, and other mitochondrial proteins required for mtDNA replication and transcription). Likewise, it was shown that maternal fat intake is associated with fatty liver in adult offspring, which is mediated through impaired hepatic mitochondrial metabolism [26].

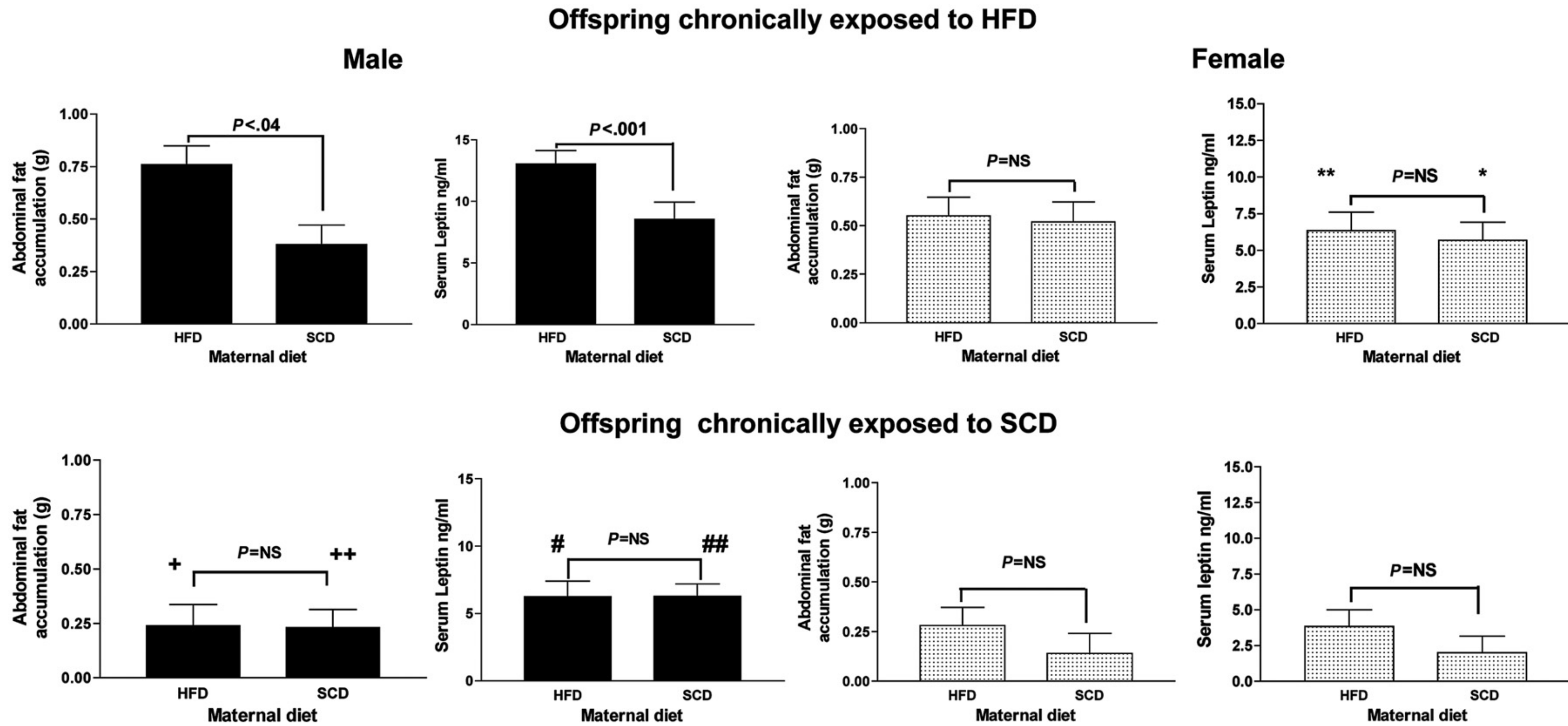

Fig. 5. The metabolic programming effect is sex specific: Male offspring of dams fed high-fat diet are hyperleptinemic and exhibit abnormal abdominal fat accumulation after exposure to a nutritional insult in adult life. Each bar represents the mean±S.E. for nine observations per group. Abdominal fat accumulation at the end of the experiment was normalized by animal length (g/cm). +P<.004 vs. male HFD offspring–HFD-fed dam and ++P<.004 vs. male HFD offspring–HFD-fed dam. #P<.002 vs. male HFD offspring–HFD fed dam and ##P<.002 vs. male HFD offspring–HFD-fed dam. **P<.0007 vs. male HFD offspring–HFD-fed dam and *P<.0009 vs. male HFD offspring–HFD-fed dam. Statistical significance was defined as P<.05. P values in each panel refer to comparison between offspring of HFD vs. SCD dam groups for each offspring sex and diet in the adult life.

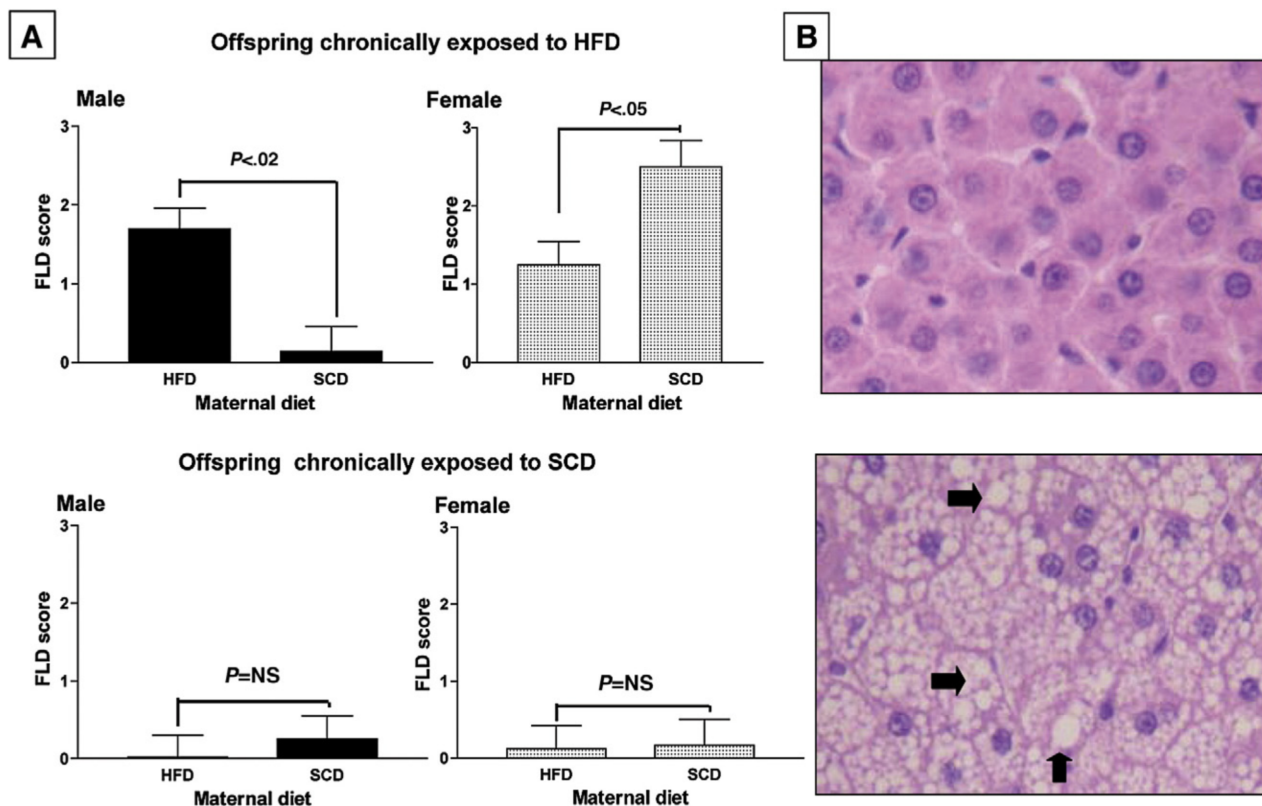

Fig. 6. (A) Maternal high-fat feeding programs fatty liver disease in male offspring chronically exposed to high-fat diet in adult life. Quantitative evaluation of steatosis scores from H&E stain of liver sections at the end of the experiment in all rats from each experimental group. Each bar represents the mean±S.E. for nine observations per group. For testing steatosis gradation (as a categorical response variable) differences, we used ANOVA or ANCOVA with ordinal multinomial distribution and probit as a link function. Pairwise mean differences were evaluated by Mann–Whitney test. Statistical significance was defined as $P<.05$ and indicates comparison between maternal diets within each sex and offspring diet group. (B) Liver histology of a representative animal from each experimental group. H&E staining of liver sections of a representative rat from each experimental group at the end of the experiment. The liver of offspring fed with SCD show normal histology and absence of fat accumulation. The liver of offspring fed with HFD show severe panlobular micro- and macrovesicular steatosis. Arrows indicate large droplet steatosis. Original magnification: 400×.

Regarding the observed changes in hepatic *Ppargc1a* expression, despite some previous evidence on the impact of the down-regulation of liver *Ppargc1a* mRNA expression on obesity-related phenotypes in experimental models of intrauterine growth restriction [27], we are able to show a novel significant interaction between intrauterine HFD programming, liver transcriptional activity of *Ppargc1a* and insulin resistance. The strong impact of the programming effect of *Ppargc1a* on the modulation of MS-related phenotypes is actually not surprising as the protein encoded by this gene is involved in the up-regulation of 649 genes linked to carbohydrate metabolism and mitochondrial functions [28]. In addition, *Ppargc1a* is involved in the control of gluconeogenesis in the liver and in the physiological control of blood pressure, cellular cholesterol homoeostasis and the development of obesity [29–31].

In agreement with our observations, a recent study on nonhuman primates showed that fetal offspring from mothers chronically consuming HFD have a threefold increase in liver triglycerides and increased evidence of hepatic oxidative stress consistent with the development of NAFLD, in addition to a reduction in the acetylated form of *Ppargc1a*, which suggests that a developing fetus is highly vulnerable to lipid excess and that exposure to this environment may directly impact on the liver [32].

Finally, a remarkable finding of our study is the protective effect of maternal programming on MS-associated phenotypes in female offspring. This surprising effect is accompanied by a significantly higher liver abundance of *Ppargc1a* transcript in females. We conjecture that the underlying mechanisms related to this sex-specific event seem to involve more than genetic or epigenetic modifications. We may speculate that this effect is closely related to the presence of sex hormones, as previous experimental evidence had shown that estrogens protect against high-fat-diet-induced insulin resistance and glucose intolerance in mice [33]. Surprisingly, estrogen-related receptors (ERRα, β and γ) are nuclear receptors closely related to the physiological functions of *Ppargc1a* [34], and previous evidence has shown an extended role for ERRα in controlling mitochondrial biogenesis [28]. In fact, *Ppargc1a* interacts with the ERRs for positive and negative control of gene expression, and *ERRα* and *Ppargc1a* are coexpressed in tissues with high energy demands [34]. In addition, the ERRs control the expression of genes directly involved not only in energy homeostasis [34,35] but also in lipid metabolism at the level of the whole organism, as previous evidence from ERRα-null mice showed them to be resistant to obesity induced by a high-fat diet [36]. In addition, a broader effect of sex chromosome complement [37] may be involved and worthy of further studies.

In conclusion, exposure to a nutritional insult in early life strongly modulates the functionality of metabolically active target tissues, such the liver. As shown in our work, this programming effect operates even during adulthood and might be able to modify the liver tissue performance and adaptation to metabolic challenges such as HFD. Moreover, this “liver metabolic imprinting” during fetal life may further contribute to the pathogenesis of adult complications such as insulin resistance and fatty liver. This study also shows that the phenotypic features were strongly related to the liver transcriptional activity of *Ppargc1a*, which showed sex-specific differences. Hence, we propose that early

intervention during pregnancy might reduce the alarming figures of MS prevalence and associated conditions in children and the adult population.

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