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Early life epigenetic programming of health and disease through DOHaD perspective

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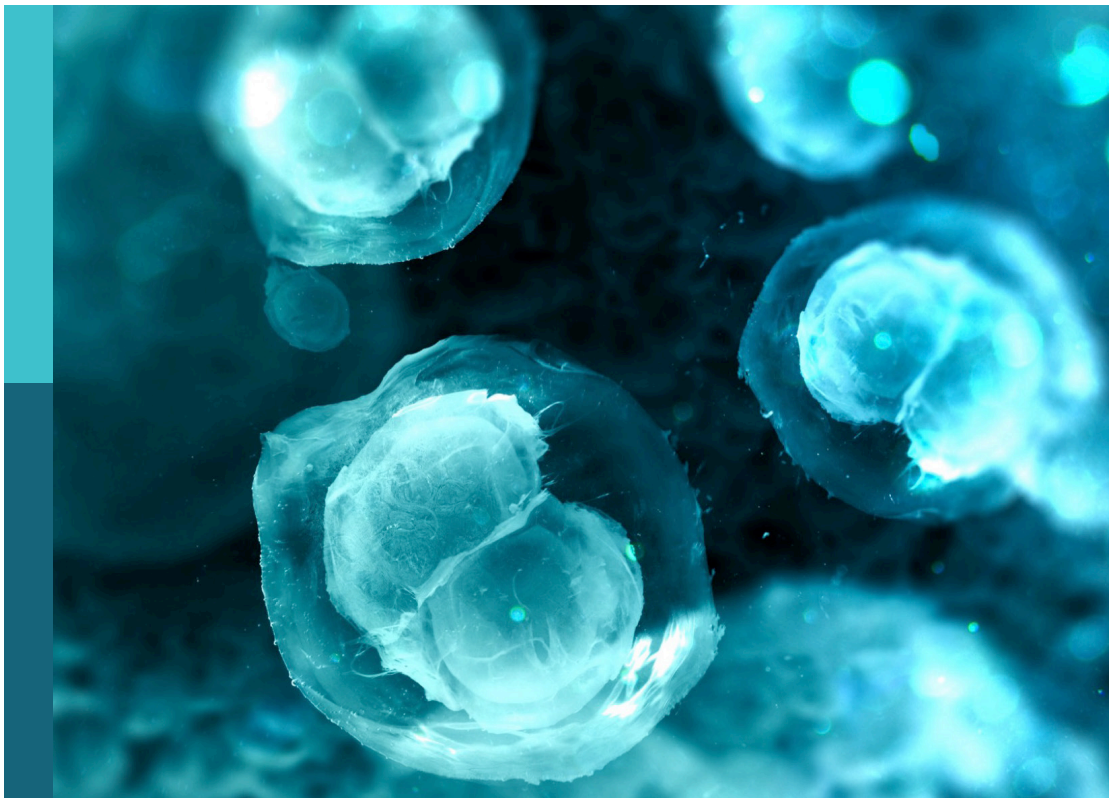

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Early life epigenetic programming of health and disease through DOHaD perspective

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Editorial: Early Life Epigenetic Programming of Health and Disease through DOHaD Perspective

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DOHAD, developmental plasticity, epigenetics, adult disease, aging

Editorial on the Research Topic
Early Life Epigenetic Programming of Health and Disease through DOHaD Perspective

Introduction

Over the past decades, the Developmental Origins of Health and Disease (DOHaD) has been consolidated as a concept asserting the causal effects of early life exposure to environmental stressors (including malnutrition, pollutants, and stress) and the global increase in non-communicable chronic diseases observed in modern society (Gluckman et al., 2010). Although multiple mechanisms have been proposed to underlie developmental programming, epigenetic processes (including DNA methylation, histone post-translational modifications, and dysregulated non-coding RNA expression) have been described as a key mechanistic framework contributing to the non-genomic heritable increase in risk disease (Treviño et al., 2020). The articles published in this Research addressed several aspects of how early life exposure to different adverse conditions may influence health and diseases throughout the life span.

Sinzato et al. demonstrated, the negative impact of diabetes combined with lifelong high-fat diet consumption on reproductive parameters in dams, while Garcia-Santillan et al., Chavira-Suárez et al., and Simino et al. explored, respectively, the role of maternal consumption of obesogenic diet on the placental expression of nutrient transporters, methylation status in umbilical cords, and miRNA expression profile in offspring liver regeneration after partial hepatectomy. The influence of parental high-fat high-sugar diet

intake on epigenetic markers and the reproductive health of male offspring was described by Sertorio et al. and Córdoba-Sosa et al. Maternal exposure to protein malnutrition was associated with the dysregulation of cell proliferation, differentiation, and impairment of epididymis development and growth (Cavariani et al.), heart fibrosis, and cardiomyocyte hypertrophy in male offspring (Folguieri et al.). An increased risk of chemically-induced mammary carcinogenesis was also reported in female offspring exposed to a maternal low protein diet (Zapaterini et al.). Wang et al., demonstrated that maternal exposure to fear maternal promoted dysregulation of the placental gene expression profile, which can contribute to placental damage and affects offspring health. Gauvrit et al. in an elegant review highlighted the association of early life exposure to stress and the development of Alzheimer's disease (AD), emphasizing the key role of epigenetic markers on the early life origins of AD.

Perspectives

Overall, the data published in this Research Topic presents new insights into the long-lasting effects of early life exposure to environmental stressors on offspring health. The promising results highlight the role of epigenetic markers as a key mechanistic framework underlying the Developmental Origins of Health and Disease and justify trials for early-life interventions to improve expectancy and quality of life.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Maternal Low-Protein Diet Deregulates DNA Repair and DNA Replication Pathways in Female Offspring Mammary Gland Leading to Increased Chemically Induced Rat Carcinogenesis in Adulthood

Joyce R. Zapaterini[1], Antonio R. B. Fonseca[1], Lucas T. Bidinotto[2,3], Ketlin T. Colombelli[1], André L. D. Rossi[4], Laura Kass[5], Luis A. Justulin[1] and Luis F. Barbisan[1]**

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Studies have shown that maternal malnutrition, especially a low-protein diet (LPD), plays a key role in the developmental mechanisms underlying mammary cancer programming in female offspring. However, the molecular pathways associated with this higher susceptibility are still poorly understood. Thus, this study investigated the adverse effects of gestational and lactational low protein intake on gene expression of key pathways involved in mammary tumor initiation after a single dose of *N*-methyl-*N*-nitrosourea (MNU) in female offspring rats. Pregnant Sprague–Dawley rats were fed a normal-protein diet (NPD) (17% protein) or LPD (6% protein) from gestational day 1 to postnatal day (PND) 21. After weaning (PND 21), female offspring (n = 5, each diet) were euthanized for histological analysis or received NPD (n = 56 each diet). At PND 28 or 35, female offspring received a single dose of MNU (25 mg/kg body weight) (n = 28 each diet/timepoint). After 24 h, some females (n = 10 each diet/timepoint) were euthanized for histological, immunohistochemical, and molecular analyses at PDN 29 or 36. The remaining animals (n = 18 each diet/timepoint) were euthanized when tumors reached ≥2 cm or at PND 250. Besides the mammary gland development delay observed in LPD 21 and 28 groups, the gene expression profile demonstrated that maternal LPD deregulated 21 genes related to DNA repair and DNA replication pathways in the mammary gland of LPD 35 group after MNU. We further confirmed an increased γ-H2AX (DNA damage biomarker) and in ER-α immunoreactivity in mammary epithelial cells in the LPD group at PND 36. Furthermore, these early postnatal events were followed by significantly higher mammary carcinogenesis susceptibility in offspring at adulthood. Thus, the results indicate that maternal LPD influenced the programming of chemically induced mammary carcinogenesis in female offspring through increase in DNA damage and deregulation of DNA repair and DNA replication pathways. Also, *Cidea* upregulation

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gene in the LPD 35 group may suggest that maternal LPD could deregulate genes possibly leading to increased risk of mammary cancer development and/or poor prognosis. These findings increase the body of evidence of early-transcriptional mammary gland changes influenced by maternal LPD, resulting in differential response to breast tumor initiation and susceptibility and may raise discussions about lifelong prevention of breast cancer risk.

Keywords: perinatal programming, maternal low protein intake, DNA repair and replication, DNA damage, risk for mammary carcinogenesis, *N*-methyl-*N*-nitrosourea, female Sprague–Dawley

INTRODUCTION

Breast cancer is one of the most common malignancy in women worldwide and the second leading cause of cancer deaths among women (Harbeck and Gnant, 2017; Ferlay et al., 2018). The well-established risk factors for breast cancer development include age, inherited genetic mutation, hormone replacement, nutritional deficiency, lifestyle, and environmental factors (Sun et al., 2017; De Cicco et al., 2019; Fahad Ullah, 2019).

Studies have shown that breast cancer susceptibility might be predetermined because of intrauterine/neonatal programming (Hilakivi-Clarke and de Assis, 2006; Fernandez-Twinn et al., 2007; Fernandez-Twinn and Ozanne, 2010; Beinder et al., 2014; da Cruz et al., 2018). Fetal programming occurs during embryonic and fetal development, a critical period in which tissues and organs are formed, and refers to the heritable changes in gene expression that can influence diseases later in life (Barker et al., 2002; Barker, 2007). Therefore, stimulus or insult at this critical period can result in developmental adaptations that produce structural, physiological, and metabolic changes, thereby predisposing descendants to chronic diseases in adulthood, including cardiovascular and metabolic diseases and cancer (Barker et al., 2002; Kwon and Kim, 2017; Herring et al., 2018). Human and animal data have shown that maternal postconception malnutrition, especially low dietary protein intake, can cause embryonic losses and intrauterine growth restriction (IUGR) that leads to hormone imbalances, metabolic disorders, and cell signaling defects (Fernandez-Twinn and Ozanne, 2010; Wu et al., 2012). In the meantime, these alterations have been associated with increased breast cancer risk (Mellemkjær et al., 2003; Ozanne et al., 2004; Diaz-Santana et al., 2020). Hence, the perinatal alterations induced by maternal low-protein diet (LPD) intake can increase the susceptibility of the epithelial mammary cells to tumor initiation induced by environmental carcinogens (Fernandez-Twinn et al., 2007; Fernandez-Twinn and Ozanne, 2010; Diaz-Santana et al., 2020).

The use of animal models to study fetal programming elucidates the relationship between maternal environment and offspring's health (Hilakivi-Clarke and de Assis, 2006). Besides, the chemically induced carcinogenesis model is an important tool to study the multistep process of mammary carcinogenesis (Russo and Russo, 1996; Russo, 2015). Using a maternal LPD model, our research group and others reported important changes in several organs, such as liver, mammary gland, pancreas, prostate, and adipose tissue (Plank et al., 2006; Fernandez-Twinn et al., 2007; Beinder et al., 2014; Santos et al., 2019; Varuzza et al., 2019; Alejandro et al., 2020; de Oliveira Lira et al., 2020). Fernandez-Twinn et al. (2007) were the first to demonstrate the adverse effects of gestational and lactational LPD on *N*-methyl-*N*-nitrosourea (MNU)–induced mammary carcinogenesis model in female offspring from Wistar rats, using a total of three 50 mg/kg body weight (b.w.) injections at 3, 4, and 5 weeks of age in a resistant rat strain. In that pioneer study, the maternal LPD resulted in female offspring with low birth weight, increased insulin-like growth factor 1 (IGF-1) and estrogen expression, and reduced postnatal ductal branching and epithelial invasion followed by compensatory mammary growth. In addition, the maternal LPD had long-term effects in offspring adulthood including the development of hyperinsulinemia, insulin resistance, diabetes mellitus type 2, and increased risk of early-onset mammary tumorigenesis induced by MNU (Fernandez-Twinn et al., 2007). In absence of carcinogen administration, most molecular findings in female offspring mammary gland were fed a gestational LPD addressed transcriptional alterations toward cell cycle control, insulin resistance, and reactive oxygen species (ROS) pathways (Zheng et al., 2012; Beinder et al., 2014). Beinder et al. (2014) observed impairment in mammary gland development in female offspring from the Wistar rats whose dams were fed an LPD, as well as identified differential regulation of genes and pathways for factors regulating cell cycle and growth. Furthermore, gestational LPD modulates p21 gene expression and histone modifications within its promoter in the mammary gland of offspring rats that can predispose the female offspring rats to the risk of developing mammary cancer later in life (Zheng et al., 2012).

Hence, these findings suggest that intrauterine and early postnatal environments, such as a maternal low protein intake, play an important role for the developmental initiation of mechanisms underlying the programming of breast cancer in adulthood. In the classic Dutch-famine study, Painter et al. (2005) observed that IUGR in the first trimester of pregnancy is associated with an earlier reproduction phase postpartum, earlier onset of menopause, and risk for breast cancer in adulthood. Also, both environmental and dietary postnatal influences are as important as fetal programming itself for mammary gland development, as previously suggested (Russo and Russo, 1996; Hilakivi-Clarke and de Assis, 2006). Based on these findings, the postnatal phase seems to play a distinct role for mammary gland development following IUGR in the rat. Thus, the combination of the chemically induced mammary carcinogenesis model and the fetal and postnatal programing animal model is an important tool to study the uterine/neonatal environmental effects on cell vulnerability to malignant

transformation. However, the molecular mechanisms involved in chemically induced mammary carcinogenesis susceptibility by maternal LPD intake, especially during gestation and lactation, are still poorly understood. Given that a poor maternal-protein diet is observed in both gestational and lactational phases, and the breast cancer prevention remains challenging in the world, understanding how a maternal-protein diet can drive the susceptibility to mammary tumorigenesis provides prevention strategies. Therefore, this study investigated the effects of gestational and lactational LPD on the gene expression of key pathways involved in mammary tumor initiation after 24 h of MNU administration, as well as on the breast cancer susceptibility in female offspring Sprague–Dawley rats, a susceptible rat strain that mimics the human disease (Russo, 2015). The findings presented here show that intrauterine and lactational protein restriction leads to early-transcriptional mammary changes (i.e., DNA repair and DNA replication pathways) followed by an increased incidence of mammary tumor later in life in female offspring challenged with an acute MNU dose in critical postnatal windows of mammary gland development.

MATERIALS AND METHODS

Animal Housing and Experimental Design

All animal procedures in this study followed the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA). This study received institutional approval from the Ethics Committee for Animal Use of the Bioscience Institute/UNESP (CEUA) (protocol 1106). Adult female (n = 60) and male (n = 30) Sprague–Dawley rats (90 days of age) were purchased from Multidisciplinary Center for Biological Research at the University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). The animals were kept in a room under a controlled temperature (22°C–25°C), relative humidity (55%), and a photoperiod (12 h), with free access to water and food.

Virgin female rats were mated overnight with established male breeders, and the detection of spermatozoa and positive cytology for estrus phase in the vaginal smear was designated as gestational day 1 (GD1). Thus, the pregnant rats were fed a normal-protein diet (NPD) (17% protein) or an LPD (6%) from GD1 to postnatal day 21 (PND 21). Normoprotein and LPDs were provided by PragSoluções (Jaú, São Paulo, Brazil). These diets have been previously described as isocaloric and normosodic based on an AIN-93G formulation (Colombelli et al., 2017; Santos et al., 2019) (**Supplementary Table S1**). To maximize lactation performance, litter size was standardized to eight pups per litter (four females and four males). After weaning, the female offspring were allocated into two groups (NPD, n = 61; and LPD, n = 61) (separated cage/group) (**Supplementary Figure S1**). In the rat prepubertal phase, the mammary gland development shows two physiologic peaks at PND 28–29 (ductal morphogenesis) and PND 34–35 (Sinha and Tucker, 1966; Russo and Russo, 1996). Thus, female offspring received a single intraperitoneal dose of 25 mg/kg b.w. of MNU (Sigma–Aldrich, St. Louis, MO, United States) dissolved in phosphate-buffered saline (PBS) acidified with acetic acid (Thompson and Adlakha, 1991) (n = 28 per diet/timepoint) at either PND 28 or 35. After 24 h of carcinogen administration, female offspring (n = 10 per diet/timepoint, one female/litter) were euthanized. The remaining animals (n = 18 each diet/timepoint, two female/litter) were followed to analyze the tumor formation until PND 250 (maximum period) and euthanized if the tumor reached ≥2 cm before PND (**Supplementary Figure S1**). Some female offspring were euthanized at PND 21 (n = 5 per diet, one female/litter) to evaluate the effects of LPD on mammary gland development prior to be switched to normal-protein diet.

For female Sprague–Dawley rats, the dose range of MNU administration is 25–80 mg/kg b.w. (Russo and Russo, 1996). In our study, the animals received a dose of MNU (25 mg/kg b.w) because it results in a low number of mammary tumors. This enabled evaluating the effects of maternal protein restriction on increasing the number of mammary adenocarcinomas in offspring. All animals were euthanized by exsanguination under sodium pentobarbital anesthesia (75 mg/kg b.w.). For each euthanasia, specific analyses were performed: whole-mount mammary gland growth (PND 21; PND 29 and PND 36) and serum estrogen and progesterone, immunohistochemistry, histopathology, and gene expression (PND 29 and 36). The tumor histology was performed in all tumor samples collected. The analysis descriptions are in the following sections. All analyses were performed comparing NPD and LPD groups on the same PND.

Blood Serum, Whole-Mount, and Immunohistochemical Mammary Gland Analyses

Blood samples (n = 5 each diet/timepoint, one female per litter) were centrifuged (2,400 *g* for 20 min), and the serum was stored at −20°C for hormonal analysis. Serum estrogen (17β-estradiol, Monobind®, 4,925-300 CA, USA; sensitivity: 6.5 pg/mL) and progesterone (Monobind®, 4,825-300, CA, USA; sensitivity: 0.105 ng/mL) were determined by colorimetric methods according to the manufacturer.

The fourth right abdominal mammary gland of female offspring (n = 5 each diet/timepoint, one female per litter) was collected and air-dried on the histological slide for 10–15 min on a clean glass slide and fixed in buffered formalin 10% for 48 h. The slides were washed in 70% ethanol, rinsed in water, and stained with carmine (1 g) and aluminum potassium sulfate dodecahydrate (2.5 g) (Sigma–Aldrich) for 4 days. Afterward, mammary gland whole mounts were dehydrated in sequential steps of ethanol (70%, 95%, and 100%), cleared in xylene, and mounted with Permount and coverslipped (Russo and Russo, 1996; Russo, 2015). Mammary gland tree was photographed using the magnifying glass at 1× magnification (Leica MZ12 DF C 420; Japan) coupled to a capture system and image analysis. Different parameters were measured for each mammary gland tree representing its outgrowth: ductal elongation, transversal growth, area, and perimeter. The total number of terminal end buds (TEBs) in the entire external margin of the mammary gland

was determined as previously described (Russo and Russo, 2006) under a microscope (Olympus Bx 53F, Japan; 20× objective).

The fourth left abdominal mammary gland (n = 5 animal/diet/timepoint, one slide/animal, one female per litter) was fixed in 10% phosphate-buffered formalin for 24 h, embedded in paraffin blocks, and cut into 5-μm-thick sections, which were stained with hematoxylin–eosin (HE) or immunohistochemically for Ki-67, ER-α, and γ-H2AX. Histological sections were placed on silanized-coated slides, deparaffinized, and rehydrated with graded alcohol. These sections were subjected to Pascal pressure chamber retrieval in a citrate acid buffer at pH 6.0 at 120°C for 30 min. Endogenous peroxidase was blocked with 3% H_2O_2 in PBS for 10 min in the dark. After washing with PBS, slides were incubated with nonfat milk in PBS for 60 min. Sections were then incubated with rabbit monoclonal anti–Ki-67, 1:100 dilution (Abcam, United Kingdom); mouse monoclonal anti–ER-α, 1:50 dilution (Invitrogen, EUA); and anti–γ-H2AX, 1:200 dilution (Invitrogen, EUA) primary antibodies in a humidified chamber (overnight, 4°C). Then, the slides were incubated with one-step horseradish peroxidase polymer (EasyPath–Erviegas, Brazil) (20 min). The reaction was visualized with 3-diaminobenzidine chromogen (Sigma–Aldrich, USA) and counterstained with Harris hematoxylin.

Apoptosis was analyzed in HE-stained slides, using morphological criteria (Elmore et al., 2016). Ki-67, ER-α, and γ-H2AX labeling indexes (LI%), and apoptosis indexes (AI%) were calculated as the number of positively marked or apoptotic epithelial cells divided by the total number of cells scored ×100 (400–500 cells/mammary gland per animal). For all histological analyses, 25 randomly selected fields were considered.

Mammary Tumor Analysis Until PND 250

After MNU administration at either PND 28 or 35, the remaining animals (n = 18 each diet/timepoint, two female/litter) were followed to analyze the tumor formation until PND 250 (maximum period) and euthanized if the tumor reached ≥2 cm before PND 250. Female offspring were examined three times per week to record the presence of gross mammary tumors and the number and location of each palpable mass in different mammary gland complexes. The body weight was analyzed at birth and PND 21, 28, 35, 50, 75, 100, 125, 150, 175, 200, 225, and 250 (statistical analysis was performed at each timepoint and compared between LPD groups vs. their respective NPD groups). Data of body weight gain (g) were obtained from PND 28 to 250 among the carcinogen-treated groups that received the MNU in the same PND. For histological analysis, tumor samples were collected and fixed in 10% phosphate-buffered formalin for 24 h, embedded in paraffin blocks, cut into 5-μm-thick sections, and stained with HE. Mammary lesions were classified according to the previously published criteria (Russo, 2015). Tumor incidence (percentage of animals with tumors) and tumor latency (time between MNU administration and appearance of the first palpable tumor per animal) were recorded for each group (Russo, 2015).

Gene Expression

Twenty-four hours after MNU administration, the fourth right abdominal mammary glands from the female offspring in NPD and LPD groups (n = 5 each diet/timepoint, one female per litter) were removed and stored at −80°C. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) followed by on-column DNA digestion. RNA samples were solubilized in nuclease-free water (Qiagen), and their concentration and integrity were evaluated on a NanoVue™ Plus (GE Healthcare) and an Agilent 2100 bioanalyzer (Agilent Technologies, Boeblingen, Germany), respectively. Equal quantities (20 ng/μL) of total RNA from each sample were reverse-transcribed to the first-strand cDNA using High-Capacity cDNA Reverse Transcription Master Mix (Life Technologies, EUA) according to the manufacturer's instruction.

RNA expression profiles were compiled using 96-well TaqMan® Array Cards (TAC)-based real-time polymerase chain reaction (PCR). The custom TAC assessed 96 genes involved in cell proliferation, DNA damage, DNA repair, and apoptosis (**Supplementary Data S1**). Actb, Pum1, and Trfc genes were used as housekeeping genes to normalize mRNA expression. Target genes were amplified using the TaqMan® Universal Mastermix II (Life Technologies, USA) by a cycling protocol of heat activation at 50°C for 1 min and denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence detection was performed on QuantStudio™ 12 K Flex Real-Time PCR System (Life Technologies). The relative expression of target genes was analyzed by the comparative Ct method (ExpressionSuite™ software; Life Technologies). This study was conducted according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR experiments) guidelines (Bustin et al., 2009).

Functional Enrichment Analysis

The deregulated genes were used to identify overrepresented gene ontology categories of biological processes and pathways with the Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.8) (available at https://david.ncifcrf.gov/tools.jsp). The functional information was assessed using UniProtKB database (available at http://www.uniprot.org/). Protein-protein interaction (PPI) networks codified by deregulated genes were generated using Metasearch STRING (v10.5.1) and visualized by Cytoscape (v3.4.0). Nomenclature of genes was established by the Rat Genome Nomenclature Committee (https://rgd.mcw.edu/nomen/nomen.shtml).

Characterization of Mammary Gland Molecular Markers With Breast Cancer Patients Using an *In Silico* Approach

For further translational insights into the relationship of molecular markers observed in female offspring mammary gland whose dams were fed an LPD with breast cancer prognostic prediction, the SurvExpress database (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp) was used for risk assessment in the BRCA-TCGA breast invasive carcinoma dataset. This tool allowed the association between the set of differentially expressed genes observed in LPD 35 group with the survival of patients with breast cancer using Cox proportional risk regression, according to the risk groups

TABLE 1 | Effects of gestational and lactational low-protein diet and MNU administration on female offspring body weight evolution. [a]

Postnatal day (PND)	Group/Treatment[b]			
	NPD		LPD	
Birth weight (g)	6.8 ± 0.6		5.78 ± 0.8[d]	
PND 21	48.4 ± 4.3		22.1 ± 4.5[d]	
	NPD 28	LPD 28	NPD 35	LPD 35
PND 28	82.8 ± 9.0	44.8 ± 8.5[d]	80.3 ± 8.1	47.8 ± 6.6[d]
PND 35	105.8 ± 6.5	73.2 ± 6.8[d]	120.5 ± 13.0	71.5 ± 10.6[d]
PND 50	177.7 ± 9.8	135.7 ± 16.2[d]	178.9 ± 14.4	142.4 ± 10.8[d]
PND 75	216.4 ± 9.6	179.9 ± 11.5[d]	231.5 ± 8.3	188.2 ± 10.1[d]
PND 100	236.8 ± 12.8	203.0 ± 14.0[d]	253.1 ± 10.0	210.9 ± 13.5[d]
PND 125	255.2 ± 15.5	216.0 ± 14.8[d]	266.1 ± 8.6	225.3 ± 15.1[d]
PND 150	265.3 ± 14.3	225.6 ± 14.2[d]	276.6 ± 7.0	233.9 ± 15.4[d]
PND 175	272.1 ± 15.9	232.9 ± 14.4[d]	283.6 ± 7.1	240.6 ± 14.4[d]
PND 200	274.1 ± 15.8	243.9 ± 13.9[d]	289.7 ± 10.0	247.1 ± 13.2[d]
PND 225	277.6 ± 16.3	246.8 ± 13.1[d]	294.9 ± 8.9	251.8 ± 12.6[d]
PND 250	284.3 ± 18.9	253.2 ± 12.7	301.3 ± 8.8	255.3 ± 16.5[d]
Body weight gain (g)[c]	205.1 ± 20.4	214.5 ± 14.0	227.5 ± 8.7	211.4 ± 16.9

[a]*Values are mean ± standard deviation.*

[b]*Normoprotein diet (17% casein). LPD: low-protein diet (6% casein); PND, postnatal day; MNU–postnatal day initiation at 28 or 35; MNU,* N-*methyl*-N-*nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal day 28 or 35.*

[c]*Difference between PND 250 and PND 28 for tumor-free animals or tumor-bearing rats with small tumors (<2 cm).*

[d]*Different from NPD, group with the same MNU–postnatal day initiation. Student* t *test (*p *< 0.001).*

estimated by an optimization algorithm. First, using the 21 deregulated genes found in the LPD 35 group in comparison to the NPD 35 group, the univariate Cox analysis was performed, and we selected the genes with ($p < 0.1$) for breast cancer prognostic prediction in a multivariate analysis. In this additional analysis, we considered $p < 0.05$ as statistically significant.

Statistical Analysis

Changes in body weight and food intake, mammary development (mammary gland outgrowth and number of TEBs), serum hormones, tumor latency, LIs (Ki-67, ER-α, and γ-H2ax), and AIs were analyzed by Student *t* test. Kaplan–Meier log-rank test was performed for comparing tumor-free animals. The percentage of different tumor phenotype (%) and tumor incidence were analyzed by χ^2 test. Statistical analysis was performed in GraphPad Prism software (version 6.01; La Jolla, CA, USA). Significant differences were assumed when $p \leq 0.05$.

For gene expression, Student *t* test was applied to perform pairwise comparisons, considering a fold change of ≥1.5. The significant overrepresented gene ontology categories and pathways were assumed when a false discovery rate was ≤0.05. In Cox proportional risk regression, we considered $p < 0.1$ and $p \leq 0.05$ for univariate and multivariate analyses, respectively.

RESULTS

Female Offspring Body Weight Is Affected by Maternal LPD

There was no significant difference between the NPD and LPD groups regarding the number of total pups/litter and the number of male/female pups per litter (gender distribution) (data not shown). Body weight at birth (PND 1) and weaning (PND 21) and body weight evolution of groups are indicated in **Table 1**. In the present study, we observed that maternal low protein restriction influenced offspring body weight, which is in line with other experimental and human studies (Fernandez-Twinn et al., 2007; Herring et al., 2018; Bautista et al., 2019; Varuzza et al., 2019; Yang et al., 2020). During the experimental period (PND 1 to PND 250), the body weight of female offspring whose dams were fed an LPD was significantly lower ($p < 0.001$) than female offspring whose dams were fed with NPD. Body weight gain was measured from PND 28 to 250. There was no significant difference in body weight gain between the LPD and NPD groups after MNU tumor initiation and NPD reintroduction after weaning (NPD 28 vs. LPD 28, $p = 0.314$) (NPD 35 vs. LPD 35, $p = 0.083$) (**Table 1**).

Maternal LPD Induces a Delay in Female Offspring Mammary Gland Development

Aiming to investigate if maternal low protein intake could induce developmental changes in a hormone-responsive organ, we evaluated the mammary gland development through whole-mount preparations. The morphometric analysis of mammary gland outgrowth and the number of TEBs are shown in **Figure 1**. In addition, representative images of whole mounts prepared from each group are shown in **Figure 2** and **Figure 3**. A significant reduction in ductal elongation and transversal growth ($p \leq 0.001$ and $p \leq 0.0023$, respectively) of the abdominal mammary gland in LPD 21 and a significant reduction ($p \leq 0.0023$) in transversal growth of LPD 28 were observed in comparison to their respective NPD groups at these timepoints (**Figure 1A**, and **Figure 2**). Besides, the mammary gland area and perimeter were also significantly lower ($p < 0.001$)

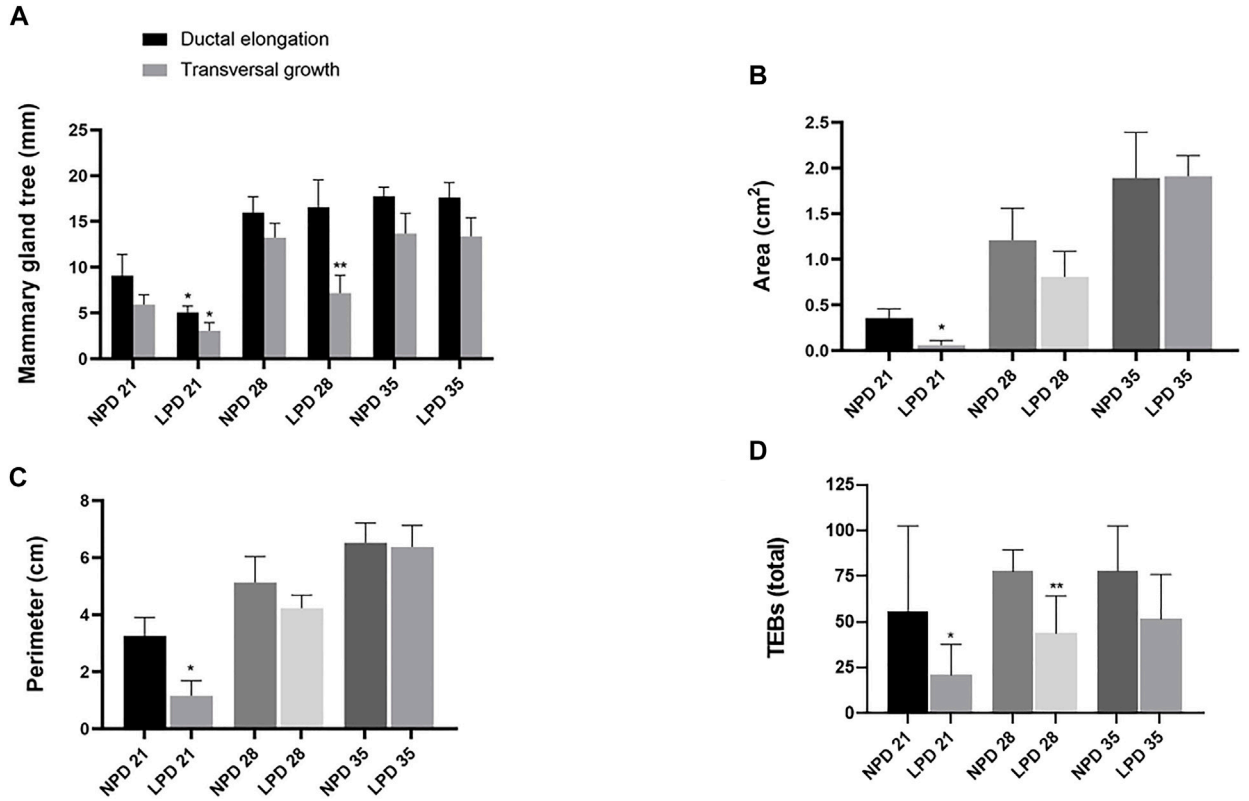

FIGURE 1 | Maternal low-protein diet programs mammary gland development of female offspring rats. Mammary gland measurements: **(A)** Ductal elongation and transversal growth (mm). **(B)** Area (cm^2). **(C)** Perimeter (cm). **(D)** The number of terminal end buds (TEBs) per field in the external margin of the mammary gland. Values expressed as mean ± standard deviation. *,**Significant different from NPD 21 and NPD 28, respectively. The differences were determined by Student *t* test ($0.001 \leq p \leq 0.049$). NPD: normoprotein diet. LPD, low-protein diet. Postnatal day of euthanasia (21) and Postnatal day of MNU administration (28 or 35). MNU, *N*-methyl-*N*-nitrosourea administration (25 mg/kg, i.p.; single dose).

in LPD 21 (**Figures 1B,C**). At PND 35, all these mammary growth parameters were similar between the NPD 35 and LPD 35 groups, demonstrating the catch-up mammary growth after feeding with adequate-protein diet (**Figure 2**). Following the mammary gland development delay observed at PND 21 and PND 28 in the LPD groups, the number of the TEBs was lower in LPD 21 ($p = 0.049$) and LPD 28 ($p = 0.010$) (**Figure 1D**, and **Figure 3**). There was no significant difference in estradiol and progesterone serum levels between the NPD and LPD groups ($p > 0.05$) (**Supplementary Figure S2**).

Mammary Tumor Susceptibility Is Increased by Maternal LPD

Considering that maternal low protein intake induces molecular changes in the mammary gland and is also associated with higher chemically induced breast cancer risk in adulthood (Fernandez-Twinn et al., 2007; Zheng et al., 2012), the modifying effects of maternal protein restriction on mammary cancer susceptibility were further investigated through two different timepoints after acute MNU administration (PND 28 or PND 35). Tumor data and percentage of tumor-free animals are shown in **Table 2** and **Figure 4**, respectively. Representative mammary tumor sections are presented in **Figure 4**. Tumor latency was similar in the LPD groups when compared with their respective NPD groups ($p = 0.748$ and $p = 0.973$ for LPD 28 and LPD 35, respectively). Although not significant ($p = 1.000$), the tumor incidence at the end of experimental period was higher in LPD 28 than in NPD 28 (44% vs. 17%), as well as in LPD 35 in comparison to NPD 35 (84% vs. 44%) (**Table 2**). However, during MNU-postinitiation days 35–175, the percentage of tumor-free rats of the LPD 35 group fell from 100% to 16%, whereas 56% of the NPD 35 group remained tumor-free ($p = 0.020$) (**Figure 4**). After histological analysis, the mammary adenocarcinomas were classified as papillary, tubular, comedo, and cribriform subtypes. The most MNU-induced adenocarcinoma showed a papillary pattern in the LPD 28 group (56%), papillary and comedo patterns in the NPD 28 group (50%), tubular pattern in the LPD 35 group (62%), and cribriform pattern in the NPD 35 group (67%) (**Table 2** and **Figure 4**).

Maternal Low Protein Intake Does Not Affect Cell Proliferation and Apoptosis But Increases ER-α Expression

As we observed a "catch-up" mammary growth and a higher mammary tumor incidence in LPD offspring, we tested whether this phenotype was due to a change in the balance between cell

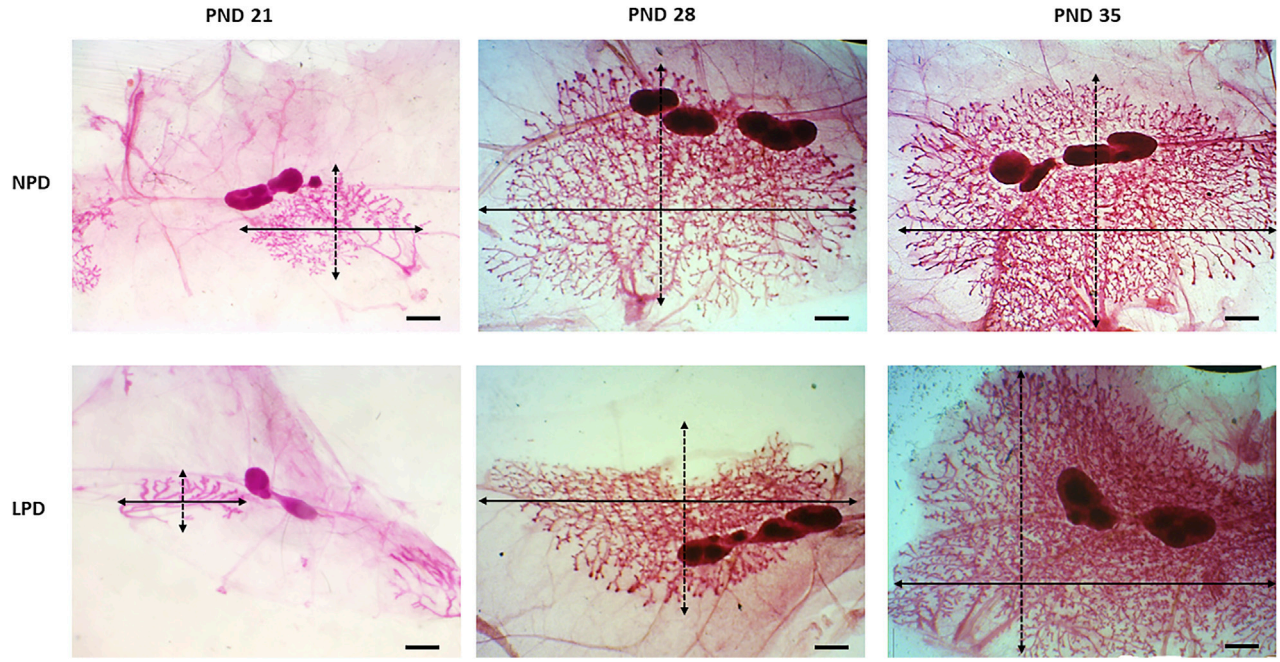

FIGURE 2 | Representative images of the whole-mount-stained mammary gland. Outgrowth measurements in each group: transversal growth (black dotted arrow) and ductal elongation (black arrow). LPD, low-protein diet; PND, postnatal day (scale bar = 20 μm).

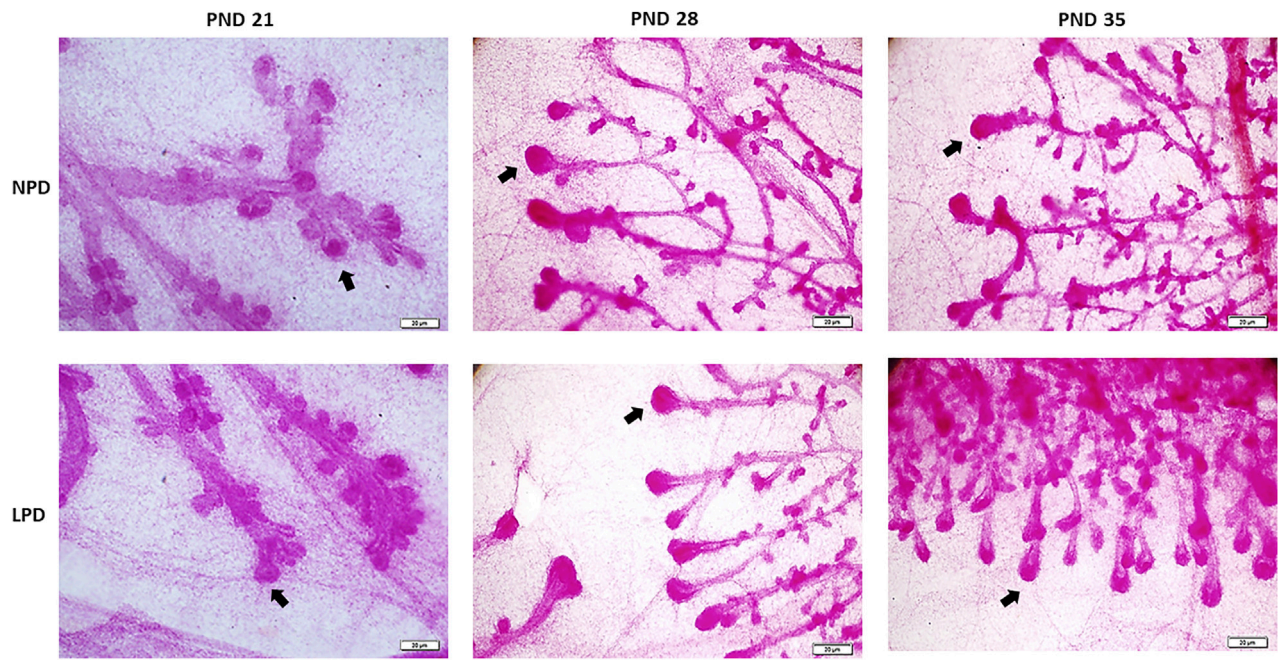

FIGURE 3 | The terminal end buds (black arrows) in the external margin of the mammary gland. NPD, normoprotein diet; LPD, low-protein diet; PND, postnatal day (scale bar = 20 μm).

proliferation and apoptosis besides the ER-α expression in mammary tissue. Cell proliferation index (Ki-67 staining) and AI were similar between the LPD and NPD groups in both timepoints ($p > 0.05$) (**Figure 5**). However, the ER-α expression in the offspring mammary epithelial tissue from the LPD 35 group was significantly higher ($p = 0.007$) than in mammary tissue of the NPD 35 group

TABLE 2 | Effects of maternal low-protein diet at gestation and lactation on MNU-induced mammary tumors in female offspring.

Parameters	Group/Treatment[a]			
	NPD 28	**LPD 28**	**NPD 35**	**LPD 35**
Number of rats	18	18	18	18
Rat bearing tumor (%)	4/18 (22%)	8/18 (44%)	8/18 (44%)	15/18 (84%)
Tumor latency (days after MNU)[b]	98.00 ± 58.00	106.75 ± 35.2	100.63 ± 56.20	99.87 ± 46.00
Total number of tumors	4	9	9	18
Histological types				
Tubular	0/4	0/9	0/9	11/18 (62%)
Papillary	2/4 (50%)	5/9 (56%)	2/9 (22%)	1/18 (5%)
Cribriform	0/4	1/9 (11%)	6/9 (67%)	5/18 (28%)
Comedo	2/4 (50%)	3/9 (33%)	1/9 (11%)	1/18 (5%)

[a]*NPD, normoprotein diet; LPD, low-protein diet; MNU,* N-*methyl*-N-*nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal day 28 or 35; PND, postnatal day.*
[b]*Values are mean ± standard deviation.*

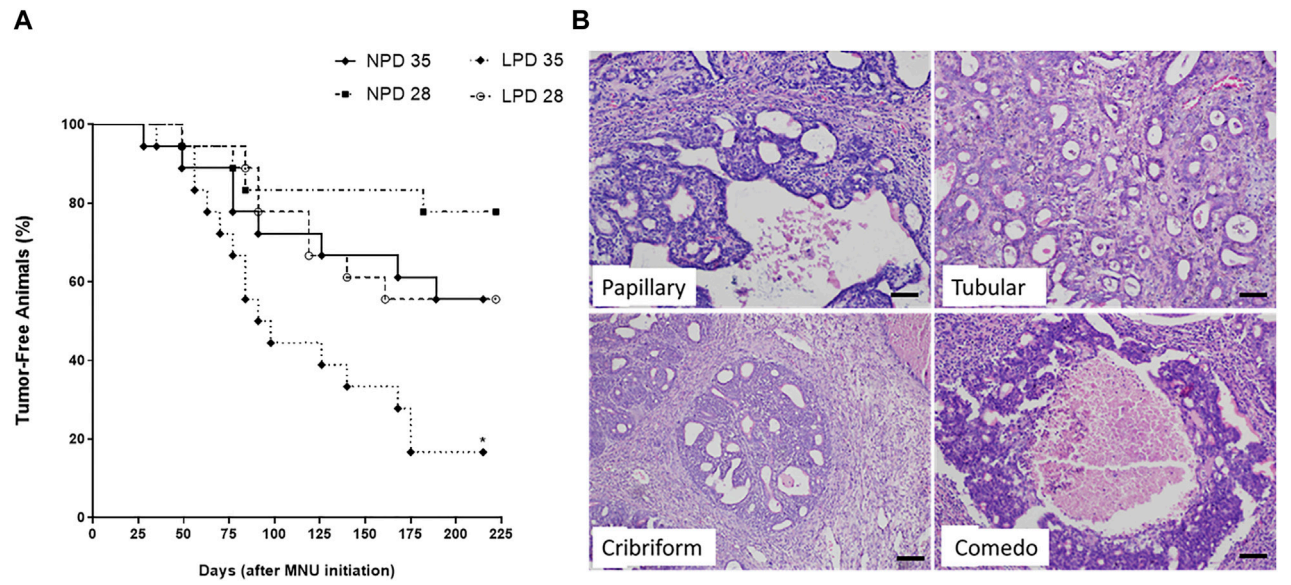

FIGURE 4 | Percentage of tumor-free animals **(A)**. Time represents the MNU-postinitiation days. NPD, normoprotein diet; LPD, low-protein diet. Postnatal day of MNU administration (28 or 35). MNU, *N*-methyl-*N*-nitrosourea administration (25 mg/kg, i.p.; single dose). *Statistically different from NPD 35 ($p = 0.020$). **(B)** Photomicrographs from tumors histological sections stained by hematoxylin–eosin (scale bar = 20 μm).

(**Figure 5**). Thus, this increase in ER-α expression may have contributed to higher tumor susceptibility in adulthood.

Maternal Low Protein Intake Alters Gene Expression in Mammary Gland After MNU Administration

Given that maternal low protein intake induces genetic changes and the higher mammary tumor incidence observed in LPD 35 female offspring, we assessed 96 genes involved in cell proliferation, DNA damage, DNA repair, and apoptosis by TAC-based real-time PCR. Twenty-four hours after MNU administration, gene expression analysis reported eight differently expressed genes between LPD 28 and NPD 28 offspring and 21 differentially expressed genes between LPD 35 and NPD 35 offspring (**Tables 3**, **4**, **Supplementary Data S1**). Of the 21 genes, 20 were downregulated and one was upregulated in the mammary tissue of LDP 35 offspring, and of the eight genes, six were upregulated and two were downregulated in the mammary tissue of LPD 28 offspring. Functional enrichment analysis demonstrated that differentially expressed genes in the LPD 35 group belong to 12 functional categories involved in several carcinogenesis-related functions, such as the regulation of cell cycle, G1/S transition of the mitotic cell cycle, apoptotic process, regulation of the apoptotic process, and response to the drug (**Table 5**). Moreover, these genes enriched 13 molecular pathways, such as microRNAs in cancer, p53 signaling pathway, cell cycle, DNA replication, base excision repair (BER), nucleotide excision repair (NER), and pathways in cancer (**Table 6**). Among these genes, *Fen1*, *Pold*, *Pole*, and *Ercc2*, which play a crucial role in DNA replication and repair, were all downregulated in LPD 35 (**Table 4**).

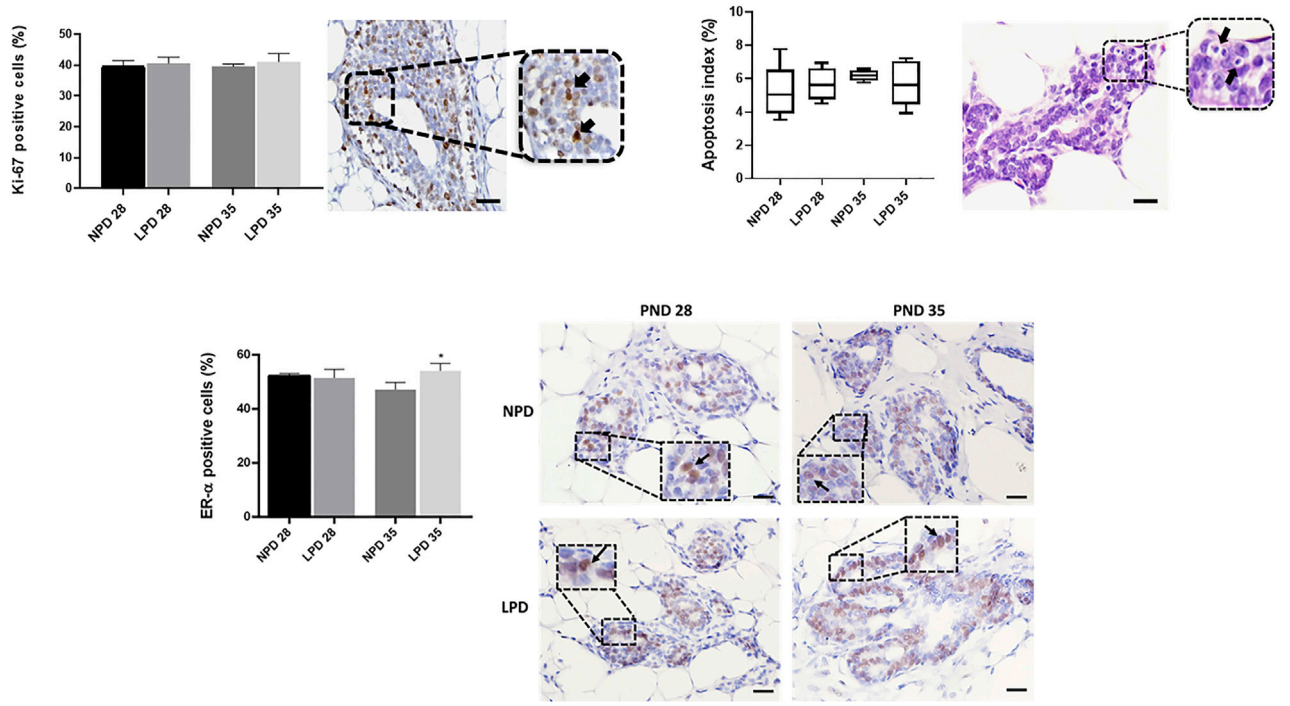

FIGURE 5 | Analysis of cell proliferation, apoptosis, and ER-α in mammary epithelial cells. Labeling index (%) and representative photomicrographs of positive mammary epithelial cells for Ki-67, apoptosis, and ER-α (black arrows) (scale bar = 20 μm). Ki-67 and ER-α values are expressed as mean ± standard deviation and apoptosis values are median and interquartile range (25%–75%). The *p* values to Ki-67 and ER-α were obtained by Student *t* test and *post hoc* Mann–Whitney rank test to apoptosis values. *Significant different from NPD35($p \leq 0.007$). NPD, normoprotein diet; LPD, low-protein diet. Postnatal day of MNU administration (28 or 35). MNU, *N*-methyl-*N*-nitrosourea administration (25 mg/kg, i.p.; single dose).

TABLE 3 | Effects of gestational and lactational low-protein diet on gene expression in female offspring mammary gland after a single MNU administration at PND 28[a].

Gene symbol	Gene name	Fold change	*p* value
Upregulated genes			
Aven	Apoptosis, caspase activation inhibitor	1.707	0.029
Cd40	CD40 molecule, TNF receptor superfamily member 5	1.785	0.032
Ercc1	Excision repair cross-complementing rodent repair deficiency, complementation group 1	1.510	0.000
Downregulated genes			
Egfr	Epidermal growth factor receptor	−1.812	0.045

[a]*Relative expression levels were determined by normalization to* Actb, Pum1, *and* Trfc, *24 h after carcinogen administration. Experimental groups were compared using Student* t *test. Fold change boundary of 1.5 and a* p *value of <0.05 were used. MNU =* N*-methyl-*N*-nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal day 28 or 35. PND = Postnatal day.*

The integrated PPI network of LPD 35 deregulated genes shows a higher number of interactions between proteins of the DNA repair, DNA replication, apoptotic process, and cell cycle control (**Supplementary Figure S3**).

Maternal LPD Increases DNA Damage in Mammary Epithelial Cells

As maternal low protein intake decreased gene expression related to DNA repair, we hypothesized that DNA damage could be higher in female offspring mammary epithelial cells whose dams were fed an LPD in relation to the counterparts whose dams were fed an NPD. Then, the immunoreactivity for phosphorylated (Ser-139 residue) histone H2A.X was evaluated in the mammary gland epithelial cells. The induction of γ-H2AX is one of the earliest events detected in cells after induction of a double-stranded DNA break and provides a sensitive, efficient, and reproducible measurement of the amount of DNA damage (Sedelnikova and Bonner, 2006). The γ-H2AX–positive cells were significantly higher in epithelial mammary cells of LPD 35 when compared with the NPD 35 group ($p = 0.042$), 24 h after a single dose of MNU administration (**Figure 6**). Thus, a higher DNA damage level and a reduced DNA repair capacity could have contributed to

TABLE 4 | Effects of gestational and lactational low-protein diet on gene expression in female offspring mammary gland after acute MNU administration at PND 35[a].

Gene symbol	Gene name	Fold change	*p* value
Upregulated genes			
Cidea	Cell death–inducing DFFA-like effector a	2.194	0.045
Downregulated genes			
Api5	Apoptosis inhibitor 5	−1.522	0.004
Apaf1	Apoptotic peptidase activating factor 1	−1.656	0.000
Atxn3	Ataxin 3	−2.160	0.000
Bax	Bcl2-associated X protein	−2.160	0.000
Ccnd1	Cyclin D1	−2.463	0.007
Ccne1	Cyclin E1	−2.597	0.002
Cd44	Cd44 molecule	−2.469	0.048
Cdc25a	Cell division cycle 25 homolog A (S. pombe)	−1.669	0.007
Dnmt1	DNA (cytosine-5-)-methyltransferase 1	−2.188	0.007
Egr1	Early growth response 1	−2.857	0.015
Ercc2	Excision repair cross-complementing rodent repair deficiency, complementation group 2	−1.773	0.002
Fen1	Flap structure–specific endonuclease 1	−1.669	0.044
Foxo3	Forkhead box O3	−1.527	0.044
Jun	Jun oncogene	−2.101	0.003
Map2k7	Mitogen activated protein kinase kinase 7	−1.869	0.007
Mapk8ip1	Mitogen-activated protein kinase 8 interacting protein 1	−3.546	0.000
Pold1	Polymerase (DNA directed), delta 1, catalytic subunit	−2.667	0.005
Pole	Polymerase (DNA directed), epsilon	−2.262	0.023
Prc1	Protein regulator of cytokinesis 1	−5.495	0.035
Skp2	S-phase kinase-associated protein 2 (p45)	−3.597	0.001

[a]*Relative expression levels were determined by normalization to* Actb, Pum1, *and* Trfc, *24 after a single carcinogen administration. Experimental groups were compared using Student* t *test. Fold change boundary of 1.5 and* p < *0.05 were used. MNU,* N-*methyl*-N-*nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal day 28 or 35; PND, postnatal day.*

TABLE 5 | Enriched biological process by differential genes expressed in the female offspring mammary gland of LPD 35 group.

	Terms	Gene name	*p* value	Fold enrichment	FDR
GO: 0000082	G1/S transition of mitotic cell cycle	*Ccnd1*, *Ccne1*, *Skp2*, *Pole*, *Cdc25a*	5.38E-07	70.76	2.32E-04
GO: 0043525	Positive regulation of neuron apoptotic process	*Egr1*, *Jun*, *Bax*, *Foxo3*, *Map2k7*	1.42E-06	55.67	3.06E-04
GO: 0051726	Regulation of cell cycle	*Jun*, *Ccnd1*, *Ccne1*, *Bax*, *Skp2*	9.60E-06	34.50	0.0014
GO: 0042493	Response to drug	*Egr1*, *Jun*, *Dnmt1*, *Ccnd1*, *Ccne1*, *Bax*, *Foxo3*	1.96E-05	11.07	0.0019
GO: 0007568	Aging	*Jun*, *Dnmt1*, *Apaf1*, *Ercc2*, *Bax*, *Foxo3*	2.25E-05	15.90	0.0019
GO: 0006915	Apoptotic process	*Ercc2*, *Cidea*, *Bax*, *Foxo3*, *Map2k7*	6.94E-04	11.41	0.0485
GO: 0042981	Regulation of apoptotic process	*Egr1*, *Apaf1*, *Cidea*, *Skp2*	9.04E-04	19.65	0.0485
GO: 0071310	Cellular response to organic substance	*Egr1*, *Ccnd1*, *Bax*	9.87E-04	61.10	0.0485
GO: 0051412	Response to corticosterone	*Ccnd1*, *Ccne1*, *Bax*	0.0011	58.26	0.0485
GO: 0000122	Negative regulation of transcription from RNA polymerase II promoter	*Egr1*, *Jun*, *Dnmt1*, *Ccnd1*, *Ccne1*, *Foxo3*	0.0013	6.72	0.0485
GO: 0045471	Response to ethanol	*Egr1*, *Dnmt1*, *Ccnd1*, *Ccne1*	0.0013	17.31	0.0485
GO: 0034644	Cellular response to UV	*Pold1*, *Bax*, *Cdc25a*	0.0014	52.19	0.0485

Enrichment analysis by DAVID (Database for Annotation, Visualization and Integrated Discovery). FDR, false discovery rate; LPD, group with maternal low-protein diet and N-*methyl*-N-*nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal 35.*

TABLE 6 | Enriched molecular pathways by differentially genes expressed in the female offspring mammary gland of LPD 35 group.

KEGG_Pathway	Terms	Gene name	*p* value	Fold enrichment	FDR
rno05161	Hepatitis B	*Jun*, *Apaf1*, *Ccnd1*, *Ccne1*, *Bax*	1.97E-04	15.49	0.007
rno05206	MicroRNAs in cancer	*Dnmt1*, *Ccnd1*, *Ccne1*, *Cdc25a*, *Cd44*	2.20E-04	15.05	0.007
rno05166	HTLV-I infection	*Egr1*, *Jun*, *Ccnd1*, *Pold1*, *Bax*, *Pole*	2.98E-04	8.94	0.007
rno04115	p53 signaling pathway	*Apaf1*, *Ccnd1*, *Ccne1*, *Bax*	4.38E-04	24.60	0.008
rno05222	Small cell lung cancer	*Apaf1*, *Ccnd1*, *Ccne1*, *Skp2*	8.30E-04	19.79	0.012
rno05203	Viral carcinogenesis	*Jun*, *Ccnd1*, *Ccne1*, *Bax*, *Skp2*	0.001	9.16	0.018
rno04722	Neurotrophin signaling pathway	*Jun*, *Bax*, *Foxo3*, *Map2k7*	0.002	13.67	0.021
rno04110	Cell cycle	*Ccnd1*, *Ccne1*, *Skp2*, *Cdc25a*	0.002	13.56	0.021
rno03030	DNA replication	*Fen1*, *Pold1*, *Pole*	0.003	35.88	0.021
rno03410	Base excision repair	*Fen1*, *Pold1*, *Pole*	0.003	34.91	0.021
rno05169	Epstein–Barr virus infection	*Jun*, *Skp2*, *Map2k7*, *Cd44*	0.004	11.64	0.026
rno03420	Nucleotide excision repair	*Pold1*, *Ercc2*, *Pole*	0.005	27.48	0.028
rno05210	Colorectal cancer	*Jun*, *Ccnd1*, *Bax*	0.008	20.18	0.048
rno05200	Pathways in cancer	*Jun*, *Ccnd1*, *Ccne1*, *Bax, Skp2*	0.009	5.44	0.050

Functional enrichment analysis by DAVID (Database for Annotation, Visualization and Integrated Discovery). FDR, False Discovery Rate. LPD, group with maternal low-protein diet and N-*methyl*-N-*nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal 35.*

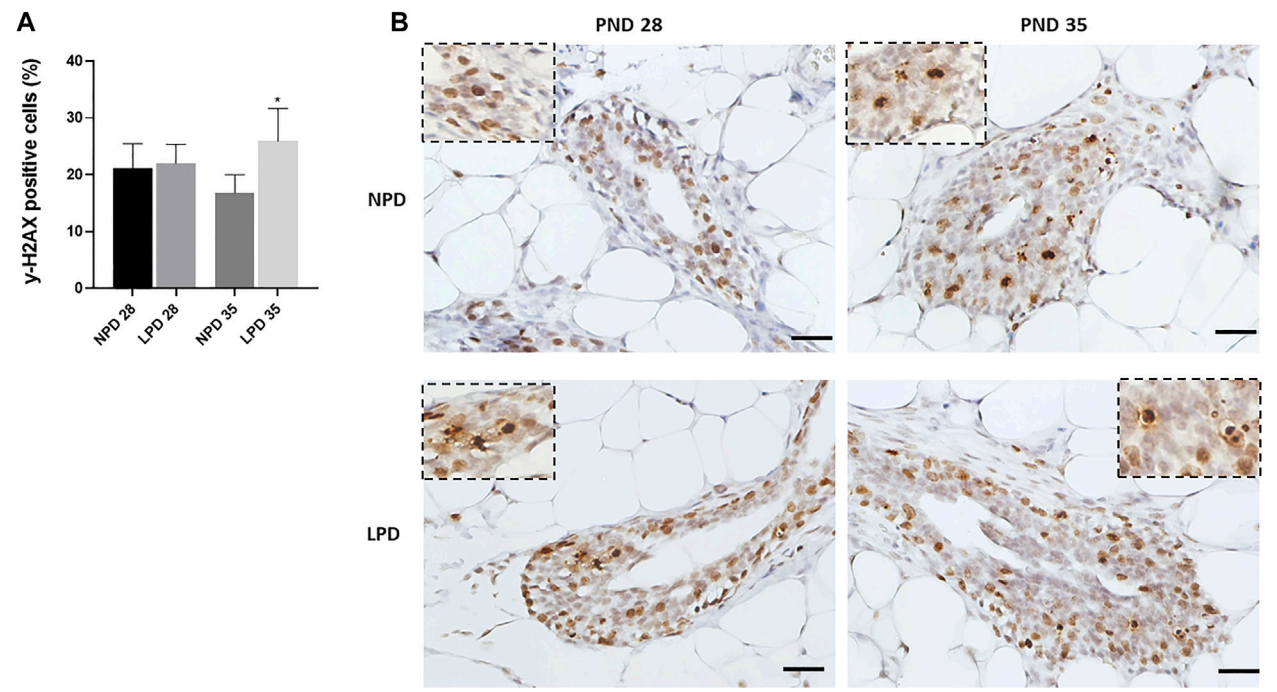

FIGURE 6 | Maternal low-protein diet increase MNU-induced DNA damage in offspring mammary epithelial cells (γ-H2AX). **(A)** γ-H2AX labeling index (%). **(B)** Representative photomicrographs of γ-H2AX-immunostained (scale bar = 20 μm). Values are expressed as mean ± standard deviation. *Statistically different from NPD 35. The *p* values ($p \leq 0.042$) were determined by Student *t* test. NPD, normoprotein diet; LPD, low-protein diet; PND, postnatal day of MNU administration (28 or 35). MNU, *N*-methyl-*N*-nitrosourea administration (25 mg/kg, i.p.; single dose).

mammary carcinogenesis susceptibility increased in LPD 35 offspring.

Prognostic Value of Deregulated Genes in Female Offspring Mammary Gland by Maternal LPD

The set of differentially expressed mammary genes in the LPD 35 group was further chosen for a translational approach involving the species-comparative *in silico* analysis on the basis of the human BRCA-TCGA invasive carcinoma dataset of patients with breast cancer including survival risk assessment. Using the 21 deregulated genes found in the LPD 35 group in comparison to the NPD 35 group, the univariate Cox analysis selected 11 genes for breast cancer prognostic prediction ($p < 0.1$). These genes were further used in multivariate Cox analysis when a significant *p* value was considered as 0.05. We identified that the high expression of *Cidea* gene predicts a lower risk of survival for

TABLE 7 | Hazard ratio and confidence intervals of human genes overlapping with differentially expressed genes in LPD 35 rat mammary tissue.

Univariate analysis			
Genes	**Hazard ratio**	**Confidence interval (95%)**	***p* value**
Cidea	1.600	1.356–1.889	2.82E-08
Fen1	0.386	0.241–0.621	0.000
Prc1	0.545	0.410–0.723	0.000
Cdc25a	0.686	0.533–0.883	0.003
Dnmt1	0.330	0.159–0.687	0.003
Pold1	0.559	0.371–0.842	0.005
Bax	0.475	0.273–0.830	0.009
Egr1	1.447	1.090–1.922	0.011
Pole	0.385	0.182–0.812	0.012
Foxo3	2.570	1.194–5.532	0.016
Ccnd1	0.721	0.503–1.034	0.076
Ercc2	0.554	0.258–1.190	0.130
Jun	1.360	0.856–2.161	0.193
Api5	0.423	0.115–1.548	0.194
Ccne1	0.896	0.712–1.129	0.352
Cd44	0.781	0.445–1.369	0.388
Map2k7	1.504	0.571–3.959	0.409
Atxn3	1.363	0.557–3.334	0.497
Mapk8ip1	1.104	0.782–1.558	0.573
Skp2	0.927	0.671–1.280	0.644
Apaf1	1.084	0.530–2.216	0.826
Multivariate analysis			
Genes	Hazard ratio	Confidence interval (95%)	*p* value
Cidea	1.5111	1.192–1.916	0.001
Foxo3	2.101	0.830–5.317	0.117
Egr1	0.787	0.529–1.171	0.238
Fen1	0.5636	0.197–1.616	0.286
Ccnd1	0.7942	0.505–1.250	0.319
Bax	1.4183	0.546–3.683	0.473
Cdc25a	1.1467	0.672–1.957	0.616
Prc1	0.8626	0.454–1.640	0.652
Pold1	0.8308	0.344–2.007	0.680
Dnmt1	1.0813	0.318–3.677	0.900
Pole	1.0113	0.346–2.957	0.984

Biomarker comparison and validation of Survival gene expression data by ServExpress software. Dataset: BRCA-TCGA breast-invasive carcinoma. Hazard ratio was estimated by fitting a CoxPH using risk group as covariate. LPD, group with maternal low-protein diet and N-*methyl*-N-*nitrosourea administered as a single intraperitoneal dose of 25 mg/kg at postnatal 35.*

patients with breast cancer (hazard ratio = 1.511 and $p = 0.001$) (**Table 7**). Thus, these results suggest that maternal low protein intake deregulates gene expression in female offspring mammary glands, which are associated with poor prognostic prediction of patients with breast cancer.

DISCUSSION

The molecular mechanisms associated with increased cancer susceptibility by maternal protein restriction using chemically induced mammary carcinogenesis models are still poorly understood. In this study, we evaluated the deleterious effects of gestational and lactational low protein intake on susceptibility to MNU-induced mammary carcinogenesis in female offspring rats, as well as on the gene expression of key pathways involved in mammary tumor initiation. Our data suggest that maternal low protein intake plays a role in programming the female offspring mammary cancer susceptibility through an increase in DNA damage and deregulation of DNA repair and DNA replication pathways after a single dose of carcinogen MNU in female SD offspring.

Maternal postconception and paternal preconception protein restriction have been associated with an increased susceptibility to early-onset chemically induced mammary tumorigenesis in the female offspring (Fernandez-Twinn et al., 2007; da Cruz et al., 2018). In the present study, even though the tumor incidence at the end of experimental period was similar between female offspring from the NPD and LPD groups, the number of tumor-free rats was significantly higher in the NPD 35 group when compared with the LPD 35 group. These results show that a maternal low protein intake increased the susceptibility to chemically induced mammary cancer in adulthood, which can be explained by morphological and molecular alterations associated with a differential response to MNU tumor initiation observed in this model. In addition to the deleterious effects of maternal protein restriction, previous studies have also shown that dietary fats are associated with an increased risk of breast cancer for mothers and female offspring (de Assis et al., 2012; de Oliveira Andrade et al., 2014; Engin, 2017; Grassi et al., 2019). Maternal high caloric intake of dietary fats/sugar increases serum estrogens during pregnancy and induces expansion of the mammary stem cell compartment during mammary development, increasing breast cancer risk in female offspring (de Assis et al., 2012; Lambertz et al., 2017).

According to the "thrifty phenotype" hypothesis, fetal malnutrition may induce physiological and/or metabolic adaptations to ensure nutrient supply to the most vital organs (such as the brain) at the expense of other organs (Hales and Barker, 2001). Studies have demonstrated that protein restriction at gestational and lactational phases impairs mammary gland development, and a compensatory growth can be observed after an adequate-protein diet supply (Fernandez-Twinn et al., 2007; Beinder et al., 2014). In the present study, the mammary gland development was also impaired in the LPD groups followed by a "catch-up" growth after an adequate-protein diet supply. Furthermore, the total number of the TEBs was reduced in LPD 21 and LPD 28 groups, which is in line with the mammary impairment and compensatory growth observed.

The mammary gland development is sensitive to steroid hormones, and an increased expression of progesterone, estrogen, and estrogen receptors has been detected in the mammary gland during the catch-up mammary growth phase after adequate-protein diet introduction (Cavalieri et al., 2006; Fernandez-Twinn et al., 2007; Arendt and Kuperwasser, 2015; Russo, 2015). Estrogen and progesterone serum levels were similar between female offspring from the NPD and LPD groups. However, the ER-α expression in epithelial mammary tissue was significantly higher in the mammary gland from the LPD 35 group when compared with the NPD 35 group. Therefore, it is possible that the high mammary epithelial ER-

α expression could have influenced the lower tumor-free animal in the LPD 35 group.

The increased susceptibility to chemically induced mammary cancer in female offspring from the LPD 35 group at adulthood may also be explained by molecular changes observed after acute response induced by MNU administration. DNA is continually exposed to endogenous and exogenous damaging agents, and failure in cell cycle regulators and DNA repair pathways drive tumor initiation (Khanna, 2015; Jeggo et al., 2016; Roos et al., 2016). In our analysis, a single MNU dose at PND 35 resulted in 21 differentially regulated genes in the LPD group. These genes belong to functional categories involved in cell cycle regulation, G1/S transition of the mitotic cell cycle, apoptotic process, and acute response to the carcinogen. Moreover, the deregulated genes also enriched some pathways such as microRNAs in cancer, p53 signaling pathway, cell cycle, DNA replication, BER, NER, and pathways in cancer. The molecular mechanisms involved in chemically induced mammary carcinogenesis susceptibility by maternal low protein intake are still poorly understood. In absence of carcinogen administration, the most molecular findings in mammary gland from female offspring with maternal protein restriction addressed transcriptional alterations toward cell cycle control, insulin resistance, and ROS pathways (Zheng et al., 2012; Beinder et al., 2014). Furthermore, these studies evaluated the effects of LPD only in gestation phase; meanwhile, the most observed scenario in population is a poor protein diet during gestation and lactation. Therefore, our gene expression profile is innovative in showing different altered pathways in female offspring mammary gland after a gestational and lactational low protein scenario and MNU exposure.

During the G1 phase of cell cycle, several metabolic, stress, and environmental signals influence the G1/S transition and cell division stop (Morgan, 2007; Rhind and Russell, 2012). Besides, to enter S phase, the cyclin-dependent kinase must be activated, and one of the mechanisms to keep Cdk2 inactive is based on limiting the supply of cyclin E (Rhind and Russell, 2012). In the present study, the *Ccnd1*, *Ccne1*, *Skp2*, and *Jun* gene expression was lower in mammary tissue from the LPD 35 group when compared with the NPD 35 group. These genes are required for cell cycle progression and for regulating the G1/S and G2/M phases transition (Wang et al., 2000; Gstaiger et al., 2001; Nakayama et al., 2010; Xu and Lin, 2018). Therefore, the low expression of these genes may be an attempt to stop cell division and avoid genomic instability. Furthermore, the p53 pathway was enriched by downregulation of *Ccne1*, *Ccnd1*, *Apaf-1*, and *Bax* genes in the LPD 35 group when compared with the NPD 35 group. The p53 pathway blocks cyclin D and cyclin E leading to cell cycle arrest and also induces apoptosis through many genes, such as *Bax* and *Apaf-1* (Harris and Levine, 2005; Joerger and Fersht, 2016; Cheok and Lane, 2017). The *Egr1* gene was also downregulated in the LPD 35 group and targets the *Pten* promoter, resulting in tumor cell apoptosis (Chen et al., 2010; Wang et al., 2021). Furthermore, *Egr1* acts on the TP53 promoter and initiates the expression of p53, which in turn activates *Egr1* forming a feedback loop (Yu et al., 2007; Wang et al., 2021). Based on these findings and considering that the AI was not different in the LPD groups, the p53 pathway may have contributed to cell cycle arrest inducing *Ccne1* and *Ccnd1* downregulation, but it was not directed toward apoptosis in response to DNA damage. On the other hand, the downregulation of *Fen1*, *Pole*, and *Pold1* genes may have impaired DNA replication accuracy and overlapped the cellular response of stopping the cell cycle (Agbor et al., 2013; Tsutakawa et al., 2014; Nicolas et al., 2016). As a consequence, impaired DNA replication accuracy could contribute to the high mammary carcinogenesis susceptibility observed in the LPD 35 group compared with the NPD 35 group.

Beyond the downregulation of *Fen1*, *Pold1*, and *Pole*, we also observed a reduction in the expression of *Ercc2* gene in the offspring mammary tissue of LPD 35 compared with NPD 35. These genes act in the cellular response to DNA damage where *Fen1* participates in the BER pathway, the *Ercc2* in the NER pathway, and the *Pole* and *Pold1* in both pathways (Balakrishnan and Bambara, 2013; Nicolas et al., 2016; Mouw et al., 2017). Therefore, these functional pathways might have impaired the DNA damage repair and contributed to the tumor initiation of mammary epithelial cells in our LPD 35 group.

Based on gene expression findings, we hypothesized that maternal protein restriction increased the DNA damage after a single dose of MNU in the female offspring mammary epithelial cells. The H2AX protein is a variant of histone H2A and, after induction of double-stranded DNA breaks, becomes phosphorylated to form gamma-H2AX (γ-H2AX). The induction of γ-H2AX is one of the earliest events detected in cells following exposure to DNA damaging agents and provides a sensitive, efficient, and reproducible measurement of the amount of DNA damage (Sedelnikova and Bonner, 2006; Mah et al., 2010). Through immunohistochemistry, we observed a higher LI for γ-H2AX epithelial cells in the mammary gland from the LPD 35 group compared with the NPD 35 group. Genomic instability is a hallmark of tumors, and mutations can arise due to increased DNA damage exposure and/or decreased DNA repair capacity (Negrini et al., 2010; Ferguson et al., 2015). Therefore, our data allow us to suggest that maternal LPD increases the DNA damage directly or/and through deregulation of DNA repair and DNA replication in the female offspring mammary epithelial cells, 24 h after MNU insult, especially at PND 35. Finally, these transcriptional and functional postnatal events resulted in increased mammary tumor susceptibility in female offspring at adulthood.

When DNA damage is not repaired, programmed cell death or apoptosis is activated to eliminate cells with extensive gene instability (Chae et al., 2016). The expression of *Apaf1*, *Bax, Egr1*, *Skp2*, *Foxo3*, *Map2k7*, and *Ercc2* genes was significantly lower in the mammary tissue from the LPD 35 group compared with the NPD 35 group. These genes participate in the positive regulation of the apoptotic process. In mammalians, the Foxo subfamily includes four genes of forkhead box-O transcription factors (Foxos), Foxo1, Foxo3, Foxo4, and Foxo6 that play a key role in cancer (Weidinger et al., 2008). Especially in humans, *Foxo*3a mediates a variety of cellular processes including apoptosis, proliferation, cell cycle progression, DNA damage, and tumorigenesis (Liu et al., 2018). In addition, the loss of *Foxo*3a expression predicted poor prognosis in human breast

cancer, probably by regulating breast cancer stem cell properties (Liu et al., 2020). Thus, considering the differential gene expression of *Foxo3* and other genes (i.e., *Apaf1*, *Bax*, *Egr1*, *Skp2*, and *Ercc2*), the negative regulation of the apoptotic process could be one of the mechanisms that led to an increased susceptibility to chemically induced mammary carcinogenesis in the LPD 35 group. However, when performing the morphological analysis to detect cells in apoptosis, there was no difference in the apoptotic index between the NPD and LPD groups. Thus, these molecular alterations could have contributed to apoptosis induction in a later postnatal phase, but without a predilection for epithelial cells initiated by MNU, resulting in higher risk for mammary carcinogenesis. As gene expression analysis was detected in whole mammary tissue (epithelium and stroma), whereas γ-H2AX, Ki-67 and apoptosis was analyzed only in the epithelial tissue, it can be considered as a limitation in this study.

The expression of *Cidea* gene has been correlated with apoptosis induction in different types of tumors, such as breast cancer (Silva et al., 2014; Bortololto et al., 2014), and is directly proportional to DNA fragmentation (Omae at al., 2012; Bortolo et al., 2017). As discussed, when the DNA damage is not repaired, programmed cell death or apoptosis is activated. When genes and proteins that positively control apoptosis are highly expressed in tumors, it seems favorable to prognosis due to the capacity for apoptosis induction. On the other hand, the increase in these genes also shows a high level of DNA damage. Thus, in some cases, such as in breast cancer, the high expression of these genes is correlated to a poor survival rate. The *in silico* analysis of human breast cancer, the TCGA dataset shows a poor prognosis of patients with *Cidea* upregulation. Similarly, we found this gene upregulated in offspring mammary gland of LPD 35 group, which may suggest that maternal LPD could deregulate genes possibly leading to increased risk of mammary cancer development and/or poor prognosis. In humans, *Cidea* is positively correlated with insulin sensitivity and healthy obesity. However, it is unknown whether Cidea causes the metabolically healthy phenotype (Abreu-Vieira et al., 2015). Even though the LPD female offspring did not develop obesity during postnatal and adult life, *Cidea* gene is linked to development of the metabolic changes and insulin resistance as described by others in this maternal malnutrition model (Zambrano et al., 2005; Fernandez-Twinn et al., 2007). This result highlights the negative impact of maternal LPD on female offspring mammary gland.

As shown in our findings, residual mammary gland growth was still observed at PND 29 (24 h after MNU administration) in the LPD group. Steroid hormones, growth hormone (GH), and prolactin are the master regulators of mammary growth. However, peptide growth factors such as epidermal growth factor (EGF), fibroblast growth factor, and IGFs and their receptors have specific roles during mammary gland development (Kleinberg and Ruan, 2008; Hynes and Watson, 2010). Each stage of mammary gland development has distinct patterns of gene expression and specific hormonal requirements that influence the cross-talk between epithelium and mesenchyme to regulate its development (Wiesen et al., 1999; Sternlicht, 2005; Hynes and Watson, 2010). Stromal–epithelial interactions are critical in determining patterns of growth, development, and ductal morphogenesis, and the EGF contributes to these stromal–epithelial interactions (Wiesen et al., 1999; Sternlicht, 2005; Hynes and Watson, 2010). In our study, the expression of epidermal growth factor receptor, a gene that regulates mammary gland ductal outgrowth with proliferative and survival roles (Sternlicht, 2005; Hynes and Watson, 2010), was significantly downregulated in mammary glands from LPD 28 group compared with NPD 28. Thus, the maternal LPD could lead to an impairment of mammary gland development in female offspring through downregulation of *Egfr* gene. The analysis of associated changes in epithelial–mesenchymal cross-talk remains to be addressed in further studies, as the mammary gland was analyzed in whole in our study.

As discussed, morphological mammary changes were observed at PND 28, whereas relevant molecular alterations and significant tumor susceptibility were observed after MNU administration at PND 35. It may be due to the differential mammary window of susceptibility to carcinogen initiation, without an important influence of mammary gland "catch-up" growth phase after NPD reintroduction.

In conclusion, the maternal low protein intake enhances MNU-induced DNA damage and deregulates DNA repair and DNA replication pathways in F1 female offspring mammary gland, which can be associated with an increase in mammary tumor development in female offspring in adulthood. These findings advance the knowledge of early-transcriptional mammary changes programmed by gestational and lactational LPD with long-term effects on mammary carcinogenesis susceptibility. Further epigenetic and proteomic studies are needed to clarify the underlying mechanisms and identify novel biomarkers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by COBEA. This study received institutional approval from CEUA (protocol 1106).

AUTHOR CONTRIBUTIONS

JRZ designed the project, performed the conceptualization, animal experimentation, methodology, analyses, interpretation of data, and wrote the manuscript. LFB designed the project, performed the interpretation of the data and, wrote the manuscript. ABF performed the methodology, analyses, and review of the manuscript. LTB performed the interpretation of the data and review of the manuscript. KTC performed the animal experimentation from gestational day 0 to PND 21 and review of the manuscript. ALDR performed the interpretation of the data and review of the manuscript. LK performed the interpretation of the data and review of the manuscript. LAJ designed the first part of the project (from

gestational day 0 to PND 21), provided the animal and dietary treatment and, review of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.756616/full#supplementary-material

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Early-Life Environment Influence on Late-Onset Alzheimer's Disease

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With the expand of the population's average age, the incidence of neurodegenerative disorders has dramatically increased over the last decades. Alzheimer disease (AD) which is the most prevalent neurodegenerative disease is mostly sporadic and primarily characterized by cognitive deficits and neuropathological lesions such as amyloid -β (Aβ) plaques and neurofibrillary tangles composed of hyper- and/or abnormally phosphorylated Tau protein. AD is considered a complex disease that arises from the interaction between environmental and genetic factors, modulated by epigenetic mechanisms. Besides the well-described cognitive decline, AD patients also exhibit metabolic impairments. Metabolic and cognitive perturbations are indeed frequently observed in the Developmental Origin of Health and Diseases (DOHaD) field of research which proposes that environmental perturbations during the perinatal period determine the susceptibility to pathological conditions later in life. In this review, we explored the potential influence of early environmental exposure to risk factors (maternal stress, malnutrition, xenobiotics, chemical factors . . .) and the involvement of epigenetic mechanisms on the programming of late-onset AD. Animal models indicate that offspring exposed to early-life stress during gestation and/or lactation increase both AD lesions, lead to defects in synaptic plasticity and finally to cognitive impairments. This long-lasting epigenetic programming could be modulated by factors such as nutriceuticals, epigenetic modifiers or psychosocial behaviour, offering thus future therapeutic opportunity to protect from AD development.

Keywords: Alzheimer disease, perinatal environment, epigenetic mechanisms, early-life stress, tau, amyloid-β

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INTRODUCTION

Alzheimer disease (AD), for which only symptomatic treatments are currently available, is a chronic neurodegenerative disorder and the most prevalence form of dementia. In 2020, there were over 50 million people worldwide living with dementia and this number is estimated to double every 20 years (World Health Organization, 2021). AD is a major health problem characterized by the progressive and irreversible development of neuronal damages, leading to the decline in cognitive functions, notably memory. AD, whose diagnosis is firmly established post-mortem, is defined by the extracellular accumulation of amyloid -β (Aβ) peptides into extracellular amyloid plaques (Checler, 1995) and the presence of neuronal neurofibrillary tangles (NFT) made up of intraneuronal fibrillar aggregates of hyper- and/or abnormally phosphorylated Tau proteins (« Tau pathology »), a common feature of other neurodegenerative disorders called tauopathies (Sergeant et al., 2008). In AD, spreading of the neurofibrillary lesions in the cortex (first enthorinal cortex, then hippocampus and lastly neocortex) fits to the progression of the

symptoms, supporting that Tau pathology is indeed instrumental in cognitive alterations (for review Colin et al., 2020). In agreement, Tau pathology development impairs various forms of synaptic plasticity and cognitive behaviors in mouse models (Polydoro et al., 2009; Hoover et al., 2010; Van der Jeugd et al., 2011; Burnouf et al., 2013; Lo et al., 2013; Van der Jeugd et al., 2013). In most cases, AD appears as a multifactorial disease resulting from the complex interaction between genetic predisposition and epigenetic environmental factors (Reitz et al., 2011; Armstrong, 2019). Epidemiological studies identified protective factors such as exercise, intellectual activities and consumption of fish or caffeine (La Rue, 2010; Flaten et al., 2014; Najar et al., 2019). By contrast, life-stresses, preceding clinical manifestation of AD, drive the progression of the disease and can exacerbate symptoms (Reitz and Mayeux, 2009; Reitz et al., 2011). In addition, apart from aging itself, cardiovascular dysfunctions such as obesity, hypertension and diabetes have been suggested as important risk factors for AD (Craft et al., 1998; Tortelli et al., 2017; Flores-Dorantes et al., 2020; Livingston et al., 2020; Tini et al., 2020). It is also notable that AD patients have been reported to exhibit altered glucose metabolism (Bucht et al., 1983; Craft et al., 1992, Craft et al., 1998; Matsuzaki et al., 2010) and even present an increased prevalence to develop type 2 diabetes (Janson et al., 2004). Presence of cognitive and metabolic alterations in AD patients fits with the "Type 3 diabetes" concept, stating that AD brain is insulin-resistant (Talbot et al., 2012; de la Monte, 2014; de la Monte et al., 2018; Nguyen et al., 2020). Although the pathophysiological mechanisms linking AD, insulin resistance and impaired glucose homeostasis remain to be clarified, it has been reported that Aβ oligomers impair insulin signalling by promoting insulin neuronal receptor internalization (Zhao et al., 2008) and inhibit insulin receptor substrate (IRS) 1 (Bomfim et al., 2012). In addition, using Tau knock-out mice, we and others showed that Tau loss-of-function, which is likely a consequence of Tau aggregation, might contribute to brain insulin sensitivity and metabolic impairments (Marciniak et al., 2017; Wijesekara et al., 2018; Wijesekara et al., 2021); an effect reversed by human Tau re-expression (Wijesekara et al., 2021). In line, the H1 Tau haplotype was associated to the glucose homeostasis in humans (Marciniak et al., 2017).

Interestingly, the pioneering epidemiological studies of David Barker in the early 90s established a relationship between intra-uterine-growth retardation (IUGR) and cardiovascular diseases in adulthood paving the way to a new field of research now known as DOHaD (for Developmental Origin of Health and Diseases) stating that environment during early life may shape the rest of our life (Barker, 2007). In accordance, numerous epidemiological studies in humans and experimental works in animals have clearly reinforced the idea that adverse perinatal environment, such as maternal stress, exposure to toxics and early-life malnutrition, has long-term consequences and may program, in the offspring, chronic adult diseases such as glucose dyshomeostasis and energy metabolism impairments but also cognitive disorders (Moody et al., 2017; Gawlińska et al., 2021b), all being encountered in AD patients. In light of these observations, altered perinatal neurodevelopment might sensitize to the occurrence of late-onset AD (Moceri et al., 2000; Landrigan et al., 2005; Miller and O'Callaghan, 2008; Modgil et al., 2014; Athanasopoulos et al., 2016) presumably *via* epigenetic mechanisms (Gapp et al., 2014; Lemche, 2018). In this review, we will summarize the most recent data suggesting that AD, that is usually associated with metabolic impairments developing at the adult stage and aging, may also date back to very early life. The identification of early mechanisms linking parental environment with modification of brain's epigenetic landscape in the offspring may offer new therapeutic strategies to prevent the progression of AD and other neurodegenerative diseases.

TAU AND AβPP DURING EARLY-NEURODEVELOPMENT

Maturation of central nervous system (CNS) and establishment of neuronal connections that vary in a region-specific manner can be divided schematically in two major developmental time-periods. The first period that occurs predominantly in the embryonic developmental stages in rodents and within the first two trimesters in humans, is dedicated to neurogenesis, neuronal migration and polarization whereas the second period covering lactation in rodents and the last trimester of gestation in humans is involved in intense neurite stabilization, synapse formation and establishment of brain circuitry (de Graaf-Peters and Hadders-Algra, 2006; Metzger, 2010; Cisneros-Franco et al., 2020). This indicates that the perinatal period covering both gestation and lactation constitutes a particularly sensitive critical time-window during which environmental perturbations may modify brain circuitry and may thus exert long-lasting effects on brain function, synaptic plasticity, homeostasis regulation and cognitive functions. Although the role of Tau and the metabolism of amyloid-β precusor protein (AβPP), the precursor to Aβ peptides, have been extensively described in the context of AD development, few studies have reported their role during early-neurodevelopment. For instance, AβPP acts as a cell adhesion molecule during different steps of neurodevelopment (Sosa et al., 2017) and may thus participate to the migration and maturation of newborn neurons as well as the formation of synapses. *Via* its large ectodomain, AβPP has been shown to bind extracellular matrix (ECM) molecules which play fundamental roles in the formation and maintenance of brain architecture during neurodevelopment and in adulthood (Dityatev et al., 2010; Mouw et al., 2014). Moreover AβPP, *via* dimerization and binding to ECM, participates to the migration and adaptation of newborn neurons to the early environment (Marín et al., 2010; Parsons et al., 2010; Cooper, 2013). Although the developmental role of Tau remains poorly understood, Tau is highly expressed in both rodent and human fetal brains with developmental changes in both splicing and phosphorylation. Resulting from an alternative splicing mechanism, six major isoforms of Tau coexist in the human brain with the presence of either 3 or 4 repeated sequences (named below as Tau-3R or Tau-4R) known as the microtubule-binding regions (Sergeant et al., 2008). Tau-3R short-isoform, being most abundant in fetal brain, has less affinity with microtubules, and increases neuronal plasticity

(Wang and Liu, 2008; Hefti et al., 2018; Hefti et al., 2019). In rats, the total level of Tau in the brain remains stable after birth and slightly increased during lactation with the appearance of larger Tau isoforms from post-natal day (PND) 5 to PND15 and the concomitant decrease of fetal Tau (Yu et al., 2009). Despite Tau knock-out mice showed moderate neurologic alterations (Ikegami et al., 2000; Lei et al., 2012), presumably due to compensatory mechanisms involving other microtubules associated proteins, *in vitro* studies in cultured mouse and hamster neurons suggested that Tau is involved in axogenesis, neurite outgrowth and neuronal circuit formation (Dawson et al., 2001; Sennvik et al., 2007; Biswas and Kalil, 2018). Recently, it has been proposed that Tau phosphorylation at microtubule domain region at early stages could protect the hippocampal circuit from overexcitation in 1-month-old 3xTg mice (a model of AD) by directly interacting with the pyramidal circuitry that spontaneously generates theta oscillations (Mondragón-Rodríguez et al., 2018). These results suggest that Tau phosphorylation at the initial stages of disease progression could represent a compensatory mechanism that mediates neuroprotection against hyperexcitability (Mondragón-Rodríguez et al., 2020). Taken together, adverse environmental outcomes during early-life which might affect AβPP metabolism and/or Tau protein might be prone to influence brain development and circuitry with long-lasting consequences.

EPIGENETIC MECHANISMS MEDIATE LONG-TERM INFLUENCE OF ENVIRONMENTAL FACTORS

Epigenetic inheritance has been defined as heritable changes in gene expression that are not encoded in the primary DNA sequence. Epigenetic processes can regulate DNA replication and repair, RNA transcription, and chromatin conformation, which influence in turn transcriptional regulation and protein translation. Epigenetic marks are partly inherited (transmission of epigenetic marks from one generation to another) but are also the signature and reflection of exposure to environments throughout life, particularly the early environment during gestation and lactation, which is a very sensitive period to both internal and external factors. Indeed, epigenetic mechanisms represent a means through which environmental factors can leave long-lasting memory of past experiences (Kundakovic and Jaric, 2017; Cirulli et al., 2020; Bellver-Sanchis et al., 2021; Breton et al., 2021). Although epigenetic modifications are often considered stable over time, epigenetic plasticity exists and may mediate adaptation to drastic environmental changes. Epigenetic mechanisms have broad actions but may mainly regulate 3 biological functions: DNA and RNA methylation, chromatin remodelling and expression of non-coding RNAs.

DNA and RNA Methylation

DNA methylation is one of the most studied and characterized epigenetic marks. It corresponds to the addition, *via* an enzymatic process, of a methyl (CH_3) group covalently linked to a cytosine residue in position 5 (5-methylcytosine, 5mC). DNA methylation that occurs mainly on palindromic CpG sequences, which can form CpG islands, takes place during early development and does not increase over time (Jang et al., 2017). However, recent studies indicate that DNA methylation can also occur on non-CpG sequences (called CpH, where H is A, C or T), especially in the brain of vertebrates (de Mendoza et al., 2021). Methylation of CpH, unlike CpG, is established *de novo* during the post-natal period, which corresponds to the maturation processes of neurons, and is increased later in life (Guo et al., 2014; Stroud et al., 2017). DNA methylation on CpG usually leads to inhibition of gene expression when it occurs at the promoter region of genes, while it increases transcriptional activity when it occurs in the transcribed regions. Although many studies suggest that CpH methylation is inhibitory, this is still debated (Jang et al., 2017). The addition of the CH_3 group is catalysed by DNA methyltransferases (DNMTs). There are 3 DNMTs, DNMT3A and DNMT3B being involved in *de novo* methylation, while DNMT1 catalyses the maintenance of methylation during DNA replication. DNA methylation, although quite stable, is a reversible mechanism mediated by the action of Ten-eleven translocation (TET) enzymes. This family of 3 isoforms catalyses the oxidation of 5mC to hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), the last two products can be excised from DNA through base excision repair, thus resulting in the reversion to demethylated cytosine (for review Lacal and Ventura, 2018). Interestingly, the activity of DNMTs and TETs is particularly high during neurodevelopment and the balance between these two families of enzymes is thought to play a crucial role in the dynamic regulation of brain genes involved in development and in cell differentiation (Tao et al., 2018; Cisternas et al., 2019). A recent study reported the methylation patterns of the Presenilin-1 gene (PSEN1), which encodes the catalytic peptide of the gamma-secretase complex (a key enzyme that cleaves the AβPP), in the brain of AD animals and humans. Interestingly, a temporal correlation between dynamic modifications in the PSEN1 CpG and non-CpG methylation patterns and mRNA expression during neurodevelopment and AD neurodegeneration has been reported (Monti et al., 2020). Moreover, several studies have shown that a decrease in TET and DNMT activity is associated with AD and cognitive impairment (Christian et al., 2020; Zhang et al., 2020; Antunes et al., 2021). Although it is likely that 5hmC represents the first step of active DNA demethylation, several data suggest that the 5hmC mark, which is enriched in adult CNS, is an active and independent epigenetic mark promoting the recruitment of factors allowing or not gene expression (Mellén et al., 2012; Shi et al., 2017; Stoyanova et al., 2021). In addition to DNA, methylation can also occur on mRNAs and lncRNAs. In this case, the addition of the methyl group takes place on adenine at position 6 (m6A) and is catalysed by a protein complex including METTL3 and METTL4 that carry the catalytic domain. This post-transcriptional modification of RNA promotes binding to other proteins, allowing pre-miR formation, translation or mRNA degradation. As with DNA methylation, this mechanism is reversible via the action of FTO and ALKBH5 (for review Zhou et al., 2020).

Chromatin Remodelling

In mammalian cells, histones interact with DNA to form chromatin. There are four types of histones: H2A, H2B, H3 and H4, which are present in duplicate to form an octamer, around which DNA is bound, forming a nucleosome. In the same way as DNA, histones can undergo several post-translational modifications (acetylation, methylation, phosphorylation, ubiquitination . . .), altering the structure of chromatin. Histone methylation, via histone methyltransferase (HMT) activity, on the N-terminal tail changes the degree of chromatin compaction, making the DNA accessible or inaccessible to transcription factors, regulating gene expression. Histone methylation can enable or repress gene expression depending on the histones methylated and the amount of methylation. The mechanism is dynamic as histones can be demethylated by histone demethylases (HDMs). Histones can also be acetylated on the lysine residues of the N-terminal tail. The addition of an acetyl group is catalysed by histone acetyltransferase (HAT), while histone deacetylases (HDACs) remove acetyl groups. Acetylation of lysines opens up chromatin, making the chromatin structure transcriptionally active with a greater access of transcriptional activators. In contrast, deacetylation compacts chromatin, preventing the binding of transcription factors, thus inhibiting gene expression (for review Bannister and Kouzarides, 2011).

Non-coding RNAs

There are two types of non-coding RNAs (ncRNAs) classified according to their size, ncRNAs below 200 nt are defined as "short" (sRNAs) and those above this length as "long" (lncRNAs). NcRNAs are single-stranded and the result of post-transcriptional maturation. Among sRNAs, microRNAs (22–25 nt), the most studied, can assemble to a protein complex and associate by complementarity with RNA segments usually on the 3' untranslated region, leading to translational repression or mRNA degradation depending on the level of complementarity. The lncRNAs, whose functions are less studied, have genes similar to the genes that code for proteins and have different modes of action by linking to DNA, RNA and proteins. Non-exhaustively, they can inhibit transcription by preventing the recruitment of the pre-initiation complex, regulate other epigenetic modifications by guiding enzymes (HDACs, HATs, HMTs, HATs, DNMTs . . .) to their site of action, activate the expression of neighbouring genes, bind to mRNA leading to mRNA degradation, bind to miRNAs limiting the action of the latter, regulate the splicing (for review Hombach and Kretz, 2016).

Although the emergence of Omics technologies has allowed to better characterize epigenetic modifications in the context of AD, these studies, that have been the subject of recent reviews (Monti et al., 2020; Coppedè, 2021; Nikolac Perkovic et al., 2021), are outside the scope of the present manuscript which is focused on the putative influence of the early environment on AD programming.

LONG-TERM CONSEQUENCES OF EARLY ENVIRONMENT ON TAU AND Aβ

Exposure to environmental agents has been extensively studied as a risk factor for the development of neurodegenerative diseases, such as AD (Killin et al., 2016; Wajman et al., 2018; Olayinka et al., 2019; Rahman et al., 2020). Recent studies suggest that exposure to cafeteria diets, pesticides, nanoparticles, air pollution and heavy metals during the perinatal period may sensitise to the development of AD in the long term, acting on the two lesions of the disease, Tau pathology and amyloid pathology, but also on neuroinflammation, which is considered as culprit component of the disease. Below, we will discuss the recent literature on the effects of perinatal exposure to different factors on Tau and Aβ.

Early-Life Exposure to Chemical Agents

Exposure to lead (Pb) during early-life has been particularly studied in the context of AD. Indeed, lead pollution is a major public health issue in developing cities. A meta-analysis using data on Pb exposure in Mexico City residents showed high blood Pb levels associated with a 5-point reduction in Intellectual quotient (IQ), and intellectual disability in children aged 0–4 years-old (Caravanos et al., 2014). Although no epidemiological study reports the effects of long-term Pb exposure, experimental studies in rodents (Bihaqi et al., 2014; Gąssowska et al., 2016) and monkeys (Bihaqi and Zawia, 2013) indicate that early exposure to Pb augments the phosphorylation of Tau, and is associated with an increase in the level of the cyclin-dependant kinase 5 (Cdk5), a major Tau kinase that is involved in abnormal phosphorylation of Tau in AD brain (Cruz and Tsai, 2004). In addition to these effects on Tau, studies also showed an increase in AβPP expression and plaque formation in the aging primate brain induced by early Pb exposure (from birth until 400 days of age). That was associated with a decrease in DNMT1 and DNMT3a activity and with a reduction in the acetylation and methylation of certain histones, resulting in a reprogramming of gene expression associated to neurotransmitter, growth factors and signal transduction pathways, suggesting an epigenetic effect (Wu et al., 2008; Bihaqi et al., 2011).

The action of bisphenol A (BPA), an endocrine disruptor used to manufacture polycarbonate plastics, is also well documented. Interestingly BPA, due to its lipophilic nature, is able to penetrate the blood brain barrier and has been found in the placenta, amniotic fluid, blood breastmilk, suggesting that it could act on foetuses/neonates during the perinatal life (Hines et al., 2015). Indeed, epidemiological studies have associated BPA exposure during early life stages with low birth weight and an increased risk of developing metabolic diseases in adulthood (Chevalier and Fénichel, 2015). In mice, maternal BPA exposure disrupts brain function and induces cognitive deficits (Tian et al., 2010) and learning-memory impairment in offspring (Xu et al., 2010). It also augments AβPP levels and Tau phosphorylation via increased activities of GSK3β and CDK5—two major Tau kinases (Sergeant et al., 2008)—but decreases insulin signalling and alters synaptic plasticity in adulthood (Fang et al., 2016; Xue et al., 2020). Prenatal BPA exposure deregulates the offspring hippocampal

transcriptome with genes associated with AD, oxidative stress and inflammation (Sukjamnong et al., 2020). In particular, it induces elevation of NF-κB protein and its AD-related target BACE1, a key enzyme involved in AβPP processing, suggesting that prenatal BPA exposure triggers neuroinflammation in the hippocampus and increases the susceptibility of AD through NF-κB. These modifications may explain the cognitive impairments observed in other studies (Negishi et al., 2004; Ryan and Vandenbergh, 2006; Wang et al., 2014), even with a single dose of BPA at PND10 (Viberg et al., 2011). Indeed, *in utero* and neonatal exposure to low dose of BPA may cause deregulation of miRNAs expression, DNA methylation levels and histone modifications (Singh and Li, 2012). A genome-wide analysis of the foetal mouse forebrain epigenome shows that exposure to low doses of BPA modifies the methylation in the promoter-associated CpG islands at several loci, which can alter the brain development (Yaoi et al., 2008). In rat, the exposure to the Di-(2-thyhexyl)-phtalate (DEHP), another endocrine disruptor, during gestation and lactation also increases the phosphorylation of Tau, without modification of AβPP level in hippocampus, and is associated with cognitive impairments in adulthood (Sun, 2014). However, so far no studies have investigated the long-term consequences of early-life BPA on epigenetic changes in the Microtubule Associated Protein Tau (MAPT, the gene encoding Tau) and AβPP genes.

Exposure to xenobiotics, man-made chemicals found in the environment but not endogenously produced, is increasingly common, and their presence in the brain during the perinatal period can alter CNS development. Polybrominated diphenyl ethers 209 (PBDE 209), a combustion inhibitor that spreads in the environment, has been found in breast milk and in consumer products. Studies in animals show that a single exposure to PBDE 209 at PND10, at the time of brain growth spurt in rodent, does not modify the level of Tau but affects CaMKII and synaptophysin levels in the hippocampus (Viberg, 2009), two proteins playing an essential role in neurodevelopment, whose alterations may likely explain impaired spontaneous behaviour seen in adulthood (Johansson et al., 2008). Similarly, exposure to another xenobiotic, the non-dioxin-like polychlorinated biphenyls (NDL-PCB), during lactation in Swiss albino mice led to a more pronounced decrease of the synaptic proteins synaptophysin and PSD95 and impairment of long-term memory when the brain is challenged by the injection of Aβ oligomers (Elnar et al., 2016).

Early-Life Exposure to High-Fat Diet

Maternal obesity has considerably increased among women of reproductive age in the last decades. Current estimates suggest that by 2025 more than 21% of women in the world will be obese, and in 2014, the estimated percentage of overweight and obesity among pregnant women was 33% in the United States (Poston et al., 2016). Cumulative data suggest that maternal obesity leads to metabolic and cognitive disorders in the offspring (Hasebe et al., 2021), which makes maternal obesity a major public health issue. However, although cognitive and metabolic impairments are two components of AD, the consequences of maternal perinatal high-fat diet on Tau and Aβ have been poorly investigated. A recent study by Di Meco *et al.* reports that a maternal high-fat diet (mHFD with 42% calories from fat) during gestation decreases total Tau and pathological Tau conformation level and increases the synaptic integrity, leading to improved cognitive performance in adult mice (Di Meco and Praticò, 2019). However, it has also been reported that a mHFD (45% fat) during gestation and lactation increases the level of Aβ peptides at the vascular level, and is associated with a modification of the neurovascular environment in the brain of adult mice, which leads to the alteration of the perivascular clearance of Aβ peptides (Hawkes et al., 2015). Although many works have looked at the epigenetic changes induced by mHFD in the programming of peripheral metabolic diseases, very few studies have looked at the consequences in the CNS of the offspring (Gawlińska et al., 2021a). For instance, it has been reported that consumption of a high-fat diet during gestation and lactation in the Wistar rat decreases the global level of histone H4 acetylation in the hippocampus at P50, leading to transcriptional repression (Gonçalves et al., 2017). Interestingly, a diet rich in grape juice has an opposite effect on the level of this epigenetic mark at PND21 and is associated with a beneficial effect on the brain with an antioxidant effect (Gonçalves et al., 2017; Schaffer et al., 2019) which could limit deleterious consequences of mHFD by reducing oxidative stress and acetylcholinesterase activity (Proença et al., 2021). However, to our knowledge, the putative consequences of mHFD on epigenetic changes in MAPT and AβPP genes have never been reported.

Early-Life Exposure to Tobacco

According to the WHO, more than 1.3 billion people worldwide smoke, about 40% of them being women. In low- and middle-income countries, where the proportion of people who smoke is highest, the prevalence of pregnant women who smoke is 2.6%, but can be as high as 30.8% in Russia (Caleyachetty et al., 2014). The deleterious effects of smoking on offspring are widely accepted and can lead to IUGR and neurodevelopmental changes (Perera et al., 2005; El Marroun et al., 2016). An epidemiological study on 471 individuals in Finland showed that male offspring of mothers who were exposed to cigarette smoke prenatally had more cognitive problems, although these effects were minor or absent in females (Ramsay et al., 2016). In mice, it has been shown that exposure to cigarette smoke during lactation increases the phosphorylation of Tau, and the levels of the 3R form of brain Tau, leading to a deregulation of the 3R/4R ratio of Tau during a critical period of brain development in 4-week-old offspring (La Maestra et al., 2011). Indeed, Tau phosphorylation but also the proportion of each isoform plays an important role in brain development, and particularly during lactation, during which synaptogenesis and axonal growth take place. Although not reported to date, these early changes could lead to neuronal dysfunction and exert long-term consequences.

Early-Life Exposure to Ionizing Radiation

Humans are routinely exposed to ionizing radiation (IR) from natural sources, and from man-made sources such as nuclear energy. IR is also increasingly used in the medical field in the diagnosis or treatment of diseases, for example in X-ray imaging. For example, in children, for the treatment of

brain tumours, exposure at a very young age can also influence the development of CNS. An epidemiological study on 3,094 Swedish males indicated that low-dose IR exposure during early human development can have a negative impact on cognitive abilities during childhood (Hall et al., 2004). An experimental study in mice reported that a single low-dose exposure of IR to PND10 animals deregulates the hippocampal and cortical proteome and transcriptome, impairs synaptic plasticity, reduces neurogenesis and leads to neuroinflammation later in life. Interestingly, miRNAs analysis showed an increased expression of miR-132 and miR-134 repressing the translation of proteins of the Rac1-Cofilin signalling pathway involved in synaptic plasticity and neurogenesis, highlighting a potential epigenetic mechanism (Kempf et al., 2014). Another team using a similar protocol demonstrated an increase in cortical Tau levels at PND11 (i.e. 1 day after exposure) and at 6 months. That was associated with a decline of long-term spatial memory when IR is combined to ketamine exposure, an anaesthesia commonly used to facilitate radiotherapy in children. Although it has not been directly assessed, these long-term consequences on Tau protein levels and cognitive deficits suggested the involvement of epigenetic mechanisms (Buratovic et al., 2014; Buratovic et al., 2018).

Early-Life Exposure to Asphyxia

Perinatal asphyxia is a defect in blood flow or gas exchange from the mother to the foetus or vice versa, occurring immediately before, during or after birth. It accounts for up to 20 cases per 1,000 births in developing countries, with mortality during the neonatal period of 15–20%, while 25% of survivors develop short- and long-term neurological disorders, associated with neonatal encephalopathy in most cases (Gillam-Krakauer and Gowen Jr, 2021). The most common diagnosis of perinatal asphyxia is addressed by chest X-ray, which, as mentioned in the previous paragraph, can be deleterious, especially in the young individual. Thus, it is important to uncover biomarkers released into the cerebrospinal fluid (CSF) during perinatal asphyxia to avoid X-ray exposure. With this idea in mind, one team evaluated the levels of Tau, pTau and Aβ42 in the CSF of newborn pigs that had undergone a perinatal hypoxia event a few hours after birth. They showed a decrease in the level of Aβ42, with no change in the level of Tau and pTau. They also observed an increase in S100B (Benterud et al., 2015), a glial marker of neuroinflammation which is also increased in CSF of infants with perinatal asphyxia (Massaro et al., 2014). In contrast, an epidemiological study reported that delivery by caesarean, known to limit hypoxia in cases of perinatal asphyxia, was associated with a reduction in cord blood Tau protein levels, indicating that Tau is modulated by blood oxygen concentration (Tunc et al., 2010) although it could not be excluded that Tau variation results from other factors associated with caesarean delivery. However, it is clear that perinatal asphyxia modified Tau, pTau and Aβ42 in the fetus/neonate, it remains to be determined if these early-life changes influence (or not) the development of late-onset AD.

LONG-TERM CONSEQUENCES OF EARLY ENVIRONMENT IN ANIMAL MODELS OF AD

Although early-life stressors exposure affects Tau and Aβ peptides levels, very few studies investigated the effects of perinatal perturbations in the pathological context of AD. This section aims at summarizing the literature on the consequences of early environment on the development of AD lesions.

Early-Life Exposure to Maternal High-Fat Diet

As previously mentioned, mHFD can have long-term deleterious effects on the levels of Tau and Aβ peptides. The consequences of mHFD have also been studied in the 3xTgAD mouse, a model of AD that develops early amyloid pathology and later Tau pathology. A first team reported that the application of mHFD (60% fat) during gestation and lactation impaired short- and long-term spatial memory and increased the number of Tau-positive neurons in the hippocampus without altering Aβ peptides level, suggesting that mHFD exacerbated Tau pathology and may sensitize to the development of AD (Martin et al., 2014). Surprisingly, using the same animal model it has also been reported that mHFD (42% fat) only applied during gestation protects from synaptic dysfunctions and associated cognitive disorders and decreases Tau and amyloid pathology by reducing the amount Aβ40 and Aβ42. This putative protective effect of the maternal diet could result from an increased gene expression of the transcriptional repressor FOXP2, which could be responsible for the decreased gene expression of Tau, CDK5 (a Tau kinase) and BACE-1 (involved in the amyloidogenic pathway) possibly via epigenetic mechanisms (Di Meco et al., 2019). It is important to note that an important limitation of this model is that the mice are homozygous for all 3 mutant alleles, and thus the observed phenotype is the result of the diet given to transgenic dams. Therefore, it is not entirely clear if changes result from the diet effect only or from the convergent impact of the diet and the transgenic phenotype of dams. Using the Tg2576 mouse, another model of AD expressing the human Swedish AβPP mutation and developing only amyloid pathology, it has been reported that a mHFD (45% fat) starting before crossing until weaning increases the level of soluble Aβ and the number of amyloid plaques. It has been suggested that these alterations could modify the extracellular matrix leading to increase Aβ plaques retention within the parenchyma (Nizari et al., 2016). These seemingly contradictory results may be explained by the different amount or types of fat used in the diets and/or by the timing of exposure of mHFD, as well as by the model used regarding amyloid pathology. Although the biological mechanisms that explain the consequences of mHFD on the development of AD are still poorly understood, recent studies suggest an important role for the gut microbiota. Indeed changes in maternal diet during the perinatal period modify both the mother's and the offspring's microbiota with maternal transmission both at delivery and through milk intake (Mueller et al., 2015). In mice, perinatal mHFD alters the short-term memory and is

associated with a deregulation of the gut microbiota in offspring, which could partly explain the observed phenotype (Sanguinetti et al., 2019). Interestingly, a high-fibre diet in mothers or offspring can restore cognitive deficits in offspring by regulating the composition of bacteria and SCFA metabolites in the long-term (Liu et al., 2021). It has been suggested that early deregulation of the microbiota may participate to cognitive impairment and to the onset of later lesions in the 3xTgAD mouse model (Bello-Medina et al., 2021). Interestingly, addition of bioactive food to the diet restores these effects and modulates the composition of the gut microbiota (Syeda et al., 2018). Together, these studies indicate that the modification of the diets or the addition of probiotics, both of which being able to modulate epigenetic mechanisms, offer the opportunity for identification of new diagnostic biomarkers and treatments (Ji and Shen, 2021).

Early-Life Exposure to Caffeine

Tea and coffee are two commonly substances consumed in the world, whose main component is caffeine, a psychoactive substance. Numerous epidemiological studies in humans and experimental studies in AD mouse models have shown the beneficial and long-term protective effects of moderate caffeine consumption in adulthood in reducing the development of lesions and related to cognitive impairment, decreasing the risk of developing AD by 65% in humans (Eskelinen et al., 2009; Flaten et al., 2014; Polito et al., 2018). Sustaining epidemiological studies, caffeine treatment of adult mice developing amyloid or Tau demonstrate positive outcomes at the pathological and behavioural levels (Arendash et al., 2006; Arendash et al., 2009; Laurent et al., 2014). In sharp contrast perinatal caffeine, by blocking adenosine A2A receptors, not only exert a detrimental impact on neuronal migration but caffeine receptors themselves strongly impact GABAergic synapse stabilization during synaptogenesis (Gomez-Castro et al., 2021). Further, perinatal caffeine strongly impacts hippocampal excitability and memory at the adult stage. Whether perinatal caffeine modulates the later development of AD pathology or has cognitive consequences remains largely unclear. However, recent data showed that perinatal caffeine consumption starting before gestation until PND15 in a transgenic mouse model of tauopathy, even not modifying the pathological load, leads to earlier long-term memory impairments together with excitatory/inhibitory synaptic drive in adulthood. These results indicate that early-life caffeine may influence brain development and exert long-lasting consequences in a Tau background (Zappettini et al., 2019).

Early-Life Exposure to Stress

Evidence indicates that chronic stress can participate to the progression of AD and other neurodegenerative disorders. In 3xTgAD mice, chronic social stress in adulthood, by regularly changing the composition of the mice in the cage, decreases BDNF levels and increases amyloid pathology and anxiety, with no change in control animals indicating a greater sensitivity to stress in mutant mice (Rothman et al., 2012). During the perinatal period, although few studies have been reported, Sierksma *et al.* have shown that chronic restraint stress performed during gestation (E1-E7) in APPswe/PS1dE9 mice model of AD induced sexual-dimorphic results in the adult offspring with males exhibiting altered spatial memory, while females have improved spatial memory associated with decreased amyloid load in the dorsal hippocampus (Sierksma et al., 2012). Although these modifications were not associated with changes in DNMT expression nor 5mC and 5hmC levels, other epigenetic mechanisms targeting histones or non-coding RNAs remain to be studied. Using the same AD animal model, it has been shown that repeated maternal separation during lactation, which is also used to induce stress during early-life, increases amyloid deposits in the hippocampus and cortex, and impairs learning and spatial memory in adulthood (Hui et al., 2017). The same experimental protocol increases Tau and amyloid pathology, decreases synaptophysin and PSD95 (two synaptic markers), impairs memory and is associated with hyperactivity of the hypothalamo-pituitary-adrenal (HPA) axis in adult male rats (Martisova et al., 2013). These observations are in agreement with the elevated glucocorticoid levels in adulthood found associated with a higher risk of developing AD, by altering glial function, Tau and amyloid lesions, and synaptic plasticity (Vyas et al., 2016). The first evidence that early-life stress could induce epigenetic programming in the rat brain came from the pioneer studies of Weaver *et al.* who showed that poor maternal care leads to increased methylation in the glucocorticoid receptor (GR) promoter which was accompanied by aberrant behaviours and altered HPA responses in adult offspring (Weaver et al., 2004). Using the 3xTgAD mice, it has been reported that handling offspring during lactation (PND2-PND9), by separating the dam from her pups for 15 min, increases maternal care of the dam towards her pups upon reunion. Increased maternal care leads to a decrease both in short-term memory decline and in amyloid load in the hippocampus, suggesting that prenatal stress could be mitigated or to have antagonistic effect by postnatal maternal care.

Recently, it has been reported in a subclinical AD-like rat model, that adult animals which were prenatally-stressed (PS) exhibited increased dysfunctions of HPA axis activity and working and long-term spatial memory as well as modified cellular organisation in CA1 hippocampal region. Interestingly, these alterations were alleviated by increased postnatal maternal care during lactation. Unfortunately, the authors did not report the putative consequences of cross-fostering in adult PS rats on Aβ plaques and NFT nor on phosphorylation and protein levels of Tau (Rostami et al., 2020).

Conclusion and Perspectives

In the present review, we provided strong evidence that early-life stress may constitute another risk factor contributing to the development of late-onset AD possibly *via* long-term influence of epigenetic mechanisms (**Figure 1**). However, important questions, which are still unanswered in the context of AD, such as the putative contribution of transgenerational epigenetic inheritance, paternal contribution and sexual dimorphism on late-onset AD development, have been overlooked. It should be kept in mind that although epigenetic

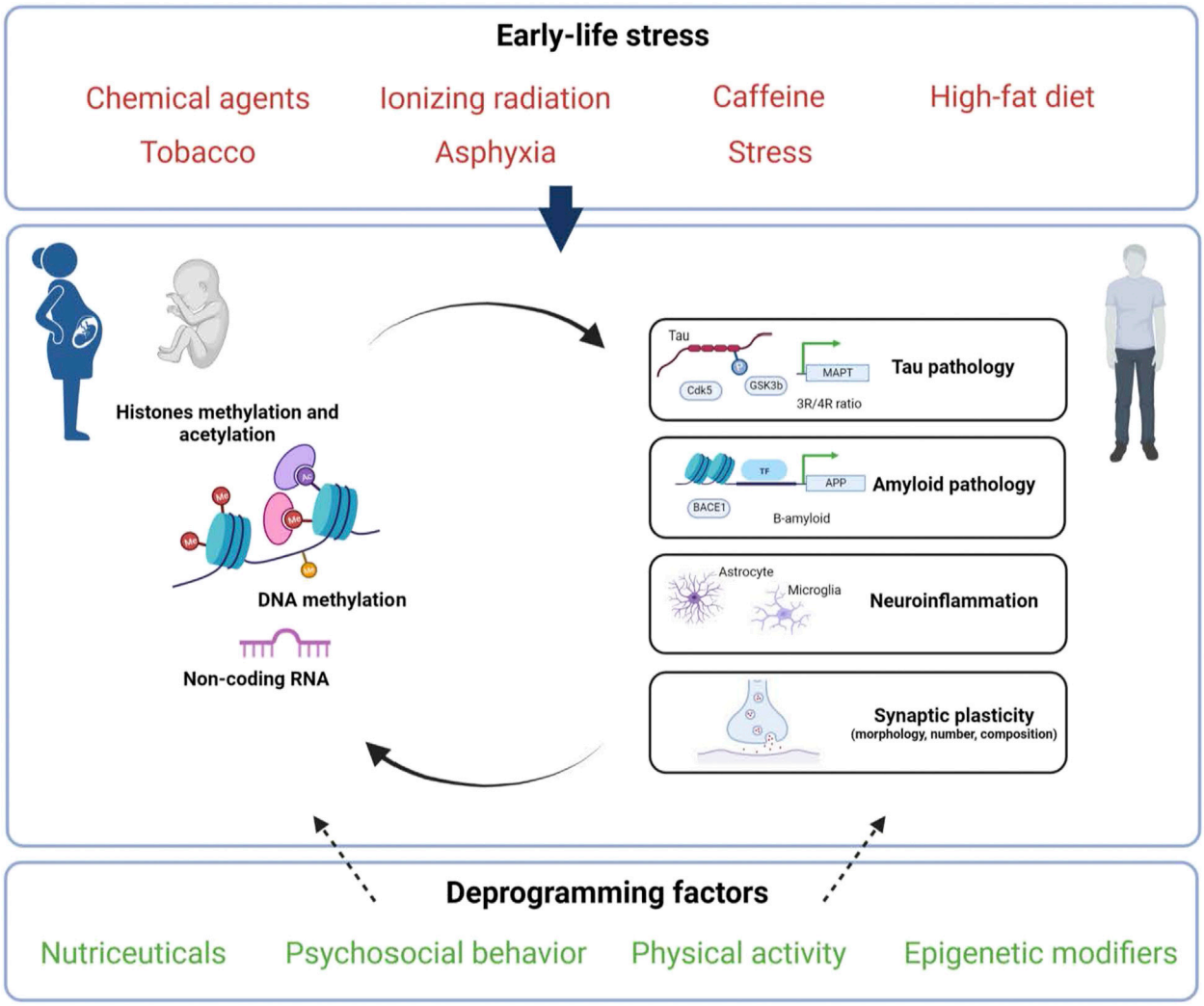

FIGURE 1 | Putative factors and epigenetic mechanisms linking early-life environment to late-onset Alzheimer's disease programming. Exposure to diverse environmental stressors (indicated in red) during perinatal life (gestation and/or lactation) may influence epigenetic mechanisms (DNA/RNA, histones and/or non-coding RNAs) and thus could have both short- and long-lasting consequences on Tau, A-β peptides, neuroinflammation and/or synaptic plasticity. These early exposures may increase the risk to develop AD but may also be counteracted by deprogramming factors (indicated in green) that could offer novel therapeutic strategies. Created with BioRender.com.

marks may be transmitted transgenerationally (Burton and Greer, 2021), the existence of multigenerational epigenetic inheritance in humans is still a matter of debate (Cavalli and Heard, 2019). Epigenetic mechanisms are indeed plastic, modifiable and reversible processes providing thus opportunities to try to reverse deleterious effects of environment *via* early-life interventional strategies (Lesuis et al., 2018). Recently, the consequences of mHFD (60% fat) have been examined in F1 (intergenerational inheritance) and F2 (transgenerational inheritance) generations of SAMP8 mice, a model of accelerated ageing leading to increased cognitive decline associated with the development of neuropathological AD hallmarks (Liu et al., 2020). The HFD applied to female mice of the F0 generation was supplemented or not with resveratrol, a natural phytoalexin known to have beneficial effects in pathological conditions, notably via its anti-inflammatory effects, and by reducing amyloid pathology and playing a role in epigenetic mechanisms (Rahman et al., 2020). Interestingly, the impairment of both short- and long-term memory and metabolic parameters (increased plasma levels of triglycerides and leptin) induced by the HFD was attenuated by resveratrol supplementation in the F0, F1 and F2 generations supporting a multigenerational effect. In addition, the level of DNA and RNA methylation (on 5mC and m6A, respectively) is increased in F1 offspring and associated with a change in the expression of enzymes involved in these epigenetic mechanisms, strongly suggesting epigenetic inheritance (Izquierdo et al., 2021). Future studies will be clearly required to understand if and how early nutritional interventions in mothers may exert beneficial effects in offspring and mitigate the long-term

deleterious effects of adverse environmental exposure on late-onset AD.

Although most of the studies focused on the consequences of maternal environmental stress in the offspring, a growing body of evidence indicates that paternal preconception stress may also have long-term effects on the offspring. Studies in rodents indicate that paternal obesity induced by HFD alters the development of the 2-cell embryo. These early events have long-lasting consequences and decrease neurogenesis and memory, and are associated with epigenetic marks changes in adulthood, including hypermethylation of BDNF promoter, a neurotrophic factor that plays a key role in brain development and function (McPherson et al., 2015; Zhou et al., 2018). It has also been reported that repeated paternal stress (Harker et al., 2015, 2018), as well as paternal BPA exposure (Luo et al., 2017), lead to behavioural alterations, associated with changes in dendritic spines and brain organisation in the offspring. In addition, paternal stress during spermatogenesis leads to altered behaviour and DNA methylation patterns with increased level in the hippocampus of both male and female PND21 offspring (Mychasiuk et al., 2013). In mice, paternal cognitive spatial training improves cognitive performance in adult offspring and is associated with an increase in the hippocampal expression of synaptotagmin, a pre-synaptic protein. This was associated with elevation of histone acetylation at the promoter of synaptotagmin in the hippocampi of both fathers and offspring as well as in fathers sperm (Zhang et al., 2017). Thus, it would be interesting to delineate the role of paternal stress in animal models of AD and to determine whether both maternal and paternal exposure to stress would have a cumulative effect. Although it would more closely mimic what happens in humans, to our knowledge, this has never been reported in the context of AD.

As previously mentioned, a large majority of works have examined the consequences of maternal stress on male offspring. To date and to the best of our knowledge, only three studies look at the consequences of early-life environment on females, even though Alzheimer's disease affects women preferentially. In the US, nearly 70% of AD patients are women, which can be explained by a longer life expectancy, but also by intrinsic biological differences, including brain organisation and function, age-associated loss of protection exerted by steroid hormones, higher inflammatory response, but also different susceptibility to genetic variations (for review Fisher et al., 2018). In addition, during early-development brain volume is higher in males, while the hippocampus is larger in females, pointing out to sexual dimorphism during a critical time-window (Kaczkurkin et al., 2019). Finally, although few studies have been reported in the context of AD, the use of mouse models led to discordant results in males and females showing the importance of studying the effects in the two sexes independently.

In conclusion, although the way by which perinatal epigenetic mechanisms act to program long-term memory and cognition remains to be clarified, it seems clear that the development of multi-omics strategies will allow molecular dissection of this fascinating new field of research. In particular, it will offer novel opportunities to identify early markers of AD and to develop novel preventive strategies to attenuate age-associated epigenetic alterations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Placental Nutrient Transporters and Maternal Fatty Acids in SGA, AGA, and LGA Newborns From Mothers With and Without Obesity

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Adverse environmental factors in early life result in fetal metabolic programming and increased risk of adult diseases. Birth weight is an indirect marker of the intrauterine environment, modulated by nutrient availability and placental transport capacity. However, studies of placental transporters in idiopathic birth weight alterations and in maternal obesity in relation to neonatal metabolic outcomes are scarce. We aimed to analyze the placental nutrient transporter protein expression in small (SGA, n = 14), adequate (AGA, n = 18), and large (LGA n = 10) gestational age term for newborns from healthy or obese mothers (LGA-OB, n = 9) and their association with maternal fatty acids, metabolic status, placental triglycerides, and neonatal outcomes. The transporter expression was determined by Western blot. The fatty acid profile was evaluated by gas chromatography, and placental triglycerides were quantified by an enzymatic colorimetric method. GLUT1 was higher in LGA and lower in SGA and positively correlated with maternal HbA1c and placental weight (PW). SNAT2 was lower in SGA, while SNAT4 was lower in LGA-OB. FATP1 was lower in SGA and higher in LGA. SNAT4 correlated negatively and FATP1 correlated positively with the PW and birth anthropometry (BA). Placental triglycerides were higher in LGA and LGA-OB and correlated with pregestational BMI, maternal insulin, and BA. Maternal docosahexaenoic acid (DHA) was higher in SGA, specifically in male placentas, correlating negatively with maternal triglycerides, PW, cord glucose, and abdominal perimeter. Palmitic acid (PA) correlated positively with FATP4 and cord insulin, linoleic acid correlated negatively with PA and maternal cholesterol, and arachidonic acid correlated inversely with maternal TG and directly with FATP4. Our study highlights the importance of placental programming in birth weight both in healthy and obese pregnancies.

Keywords: placenta, maternal obesity, nutrient transporters, fatty acids, birth weight

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INTRODUCTION

Birth weight is an indirect marker of intrauterine growth and nutrition. Birth weight alterations have been associated with adverse perinatal effects and risk of disease in adulthood. Both extremes of birth weight, either SGA (small gestational age) or LGA (large gestational age), have an increased risk of developing diabetes mellitus type 2, hypertension, obesity, insulin resistance, prostate cancer, and cardiovascular diseases in adult life (Das and Sysyn, 2004; Monasta et al., 2010; Kruger et al., 2017; Fall and Kumaran, 2019; Sharma et al., 2020).

Birth weight is influenced by several maternal factors such as genes, nutritional and hormonal state, and the capacity of nutrient transport through the placenta. Thus, availability of nutrients such as glucose, amino acids, and fatty acids from the mother is determinant for adequate fetal growth (Jones et al., 2007; Hayward et al., 2016).

Glucose is the main cellular energy substrate for the fetus, which contributes directly with intrauterine development. Glucose transport *via* the placenta occurs by facilitated diffusion, trough concentration gradient from the mother to fetus, or by the glucose transporter (GLUT) family (Jansson et al., 1993; Prendergast et al., 1999). GLUTs are proteins composed of 12 transmembrane domains, of approximately 500 amino acid residues in total, which are encoded by the *SLC2* gene family. The main isoforms that have been detected in the human placenta are GLUT1, GLUT3, GLUT4, and GLUT9, having each one a specific function during glucose transport and exchange (Arnott et al., 1994; Illsley, 2000; Bibee et al., 2011; Mueckler and Thorens, 2013). The protein expression of GLUT1 in the placenta has been positively associated with the birth weight of newborns from women with obesity without type 2 diabetes mellitus (Acosta et al., 2015). Decreased GLUT1 protein has also been reported in the placenta of women with preeclampsia (Lüscher et al., 2017). On the other hand, the protein expression of GLUT3 is higher in primary trophoblast cultures of placentas from IURG pregnancy (Janzen et al., 2013) and lower in placentas from women with gestational diabetes mellitus (Zhang et al., 2016).

Similar to glucose, amino acids have specific functions for adequate fetal development as they are precursors for structure proteins and non-proteic substances such as polyamines, nitric oxide, neurotransmitters, and purine and pyrimidine nucleotides (Grillo et al., 2008). Placental amino acid transfer to the fetus is mediated by two coordinated systems, termed system A and L. System A transports nonessential neutral amino acids by a sodium-dependent mechanism, comprising the protein transporters SNAT1, SNAT2, and SNAT4, encoded by the *SLC38A* gene family. System L consists of a heterotrimeric complex, with the L-amino acid transporter LAT1 or LAT2, together with a heavy chain 4F2hc/CD98 protein, and exchanges essential amino acids in exchange for nonessential ones, in a sodium-independent fashion (Vaughan et al., 2017; Dumolt et al., 2021). Proteins of both systems have been detected in the human placenta, mainly in the microvillous membranes facing the maternal side (Vaughan et al., 2017). System A activity and the SNAT protein expression in the human placenta, but not in the LAT expression or system L, have been found decreased in IUGR (Shibata et al., 2008; Chen et al., 2015) and correlated with the birth weight in normal and obese mothers (Jansson et al., 2013; Gaccioli et al., 2015).

Long-chain fatty acids are essential for the fetal growth, serving as eicosanoid precursors, forming cellular plasmatic membrane, and mediating the genic expression. Furthermore, 50% of the neural tissue is composed by lipids, most of which are long-chain fatty acids. An increment of the total amount of fatty acids is seen throughout pregnancy, mainly long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid 22:6n-3 (DHA) and arachidonic acid 20:4n-6 (AA), which increase 23 and 51%, respectively (Al et al., 2000). The human body is not capable of producing fatty acids with a double bond in carbons 3 and 6, which is why the intake of essential fatty acids into diet, such as linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3), is required, in order to ensure adequate concentrations of their corresponding LC-PUFA derivatives (Haggarty, 2004; Duttaroy and Basak, 2020).

Fatty acid transport into the cell occurs by passive diffusion and through protein binding. Placental fatty acid transporters include FAT/CD36 (fatty acid translocase), FABPpm (plasma membrane fatty acid-binding protein), p-FABPpm (fatty acid-binding protein in the plasma membrane of the placenta), and FATP-1 to-6 (fatty acid-transporting proteins). Particularly, FATPs, a group of transmembrane proteins encoded by the *SLC27A* gene family, are located both in the MVM and in the BM, and their overexpression has been related to an increase in fatty acid internalization, presumably long-chain fatty acids, even at low concentrations (Larqué et al., 2011; Duttaroy and Basak, 2020). FATP1 (Coe et al., 1999) and FATP4 (Hall et al., 2005) are the only transporters that also exhibit the acyl-CoA-synthetase activity, which converts the transported fatty acids into acyl-CoA metabolites, preventing their efflux. It has been suggested that this transport is crucial for increased beta-oxidation of fatty acids (Duttaroy and Basak, 2020). A positive correlation between FATP1 and FATP4 placental mRNA levels with maternal plasma DHA levels and with umbilical cord phospholipid DHA has been found (Larqué et al., 2006). Also, a higher placental protein expression of FATP1 and FATP4 has been observed in mid-gestation in obese ewes compared to lean ewes (Zhu et al., 2010). Likewise, the FATP4 mRNA and protein expression are lower in placentas of women with obesity (Mishra et al., 2012).

Most previous investigations are focused on the study of nutrient transporters' functions in the human placenta with pregnancy complications, such as preeclampsia, and gestational diabetes mellitus. Therefore, the aim of the present study was to evaluate the expression of glucose, amino acid, and fatty acid transporters in placentas of women without pregnancy pathologies, with or without obesity, and their relationship with maternal fatty acids and metabolic status. We hypothesize that there is a differential protein expression of the nutrient transporters in placentas of SGA, AGA, and LGA neonates, as well as a relationship with birth weight.

MATERIALS AND METHODS

Design and Study Population

We performed a comparative, cross-sectional, and descriptive study. Samples from women without metabolic or chronic

diseases before or during pregnancy from a previous cohort, recruited from the General Hospital of Leon, Guanajuato, Mexico, were analyzed. (Lazo-de-la-Vega-Monroy et al., 2020). The study was approved by the ethics committees of the University of Guanajuato and the corresponding health institutions (HGL-GTSSA002101-337 and CIBIUG P-23-2015 and P-43-2017). Women were recruited at the moment of delivery, and the informed consent was accepted before sample collection. Clinical and anthropometrical data were obtained from medical records and direct interview with the participants. For the neonates, anthropometrical measurements were performed according to the hospital's standardized procedures by trained health staff and were taken from the medical record.

Only samples from pregnant women between 18 and 35 years old at term (>36 weeks of gestation), with singleton pregnancies and without diabetes mellitus, gestational diabetes, hypertension, antiphospholipid antibody syndrome, preeclampsia, alcoholism, smoking, IUGR diagnosis, or fetal distress, were included.

The gestational age was determined by the Capurro method, and neonates were classified according to national birth weight tables adjusted for gestational age and sex (Flores-Huerta and Martínez-Salgado, 2012; Norma Oficial Mexicana NOM-007-SSA2-1993, 1993). A total of 51 samples were divided according to birth weight into the following groups: SGA (small for gestational age) n = 14, AGA (adequate for gestational age) n = 18, LGA (large for gestational age) n = 10, and LGA-OB (large for gestational age products of mothers with obesity) n = 9. Clinical and anthropometric data, as well as the plasma from women and cord blood, were analyzed for the selected samples.

Sampling Methods

Maternal blood samples were obtained during labor within 3–8 h of previous fasting. Placental and umbilical cord blood samples were collected immediately after delivery. A placental tissue of 5 × 5 was taken halfway between the cord insertion and the placental border within 30 min after delivery and was frozen in dry ice until use. Serum of the maternal peripheral blood and arterial umbilical cord blood samples was centrifuged, aliquoted, and stored at −20°C.

Biochemical and Hormonal Assays

Biochemical variables in mother and umbilical cord blood (glucose and lipid profile) were measured by colorimetric enzymatic methodology (Spinreact). Maternal HbA1c detection was performed by using a cation exchange resin kit (Eagle diagnostics). Neonatal and maternal insulin concentrations were measured using an ultra-sensitive ELISA Kit (ALPCO). The HOMA IR Index was calculated by the following formula: Fasting insulin (μU/ml) × fasting glucose (mmol/L)/22.5.

Placental Nutrient Transporters Protein Expression

Approximately, 150 mg of the placental tissue was taken for subsequent protein extraction with 300 μl of lysis buffer, which contained 30 μl of the protease inhibitor (MC Roche), 100 mM of Tris-HCL, 100 mM of NaF, 1 mM of disodium EDTA, 250 mM of saccharose, 150 mM of NaCl, 10 mM sodium orthovanadate, 10 mM NaPPi, and NP-40 at 1%. The samples were homogenized with a polytron and were incubated on ice for 2 h. The extracts were homogenized with a vortex for 30 min and centrifugated at 10,000 × *g* for 15 min at 4°C. The supernatant was collected, and the protein concentration for each homogenate was measured by the Lowry method using a BSA standard curve. Protein separation was performed by electrophoresis in 10% SDS-PAGE gels for GLUT1, GLUT3, SNAT1, SNAT4, and tubulin proteins. SDS-PAGE gels at 8% for SNAT2, FATP1, and FATP4 proteins were used. Proteins were transferred to nitrocellulose membranes (Hybond C Super, Amersham Pharmacia Biotech). The membranes were blocked with a 5% skim milk solution during 2 h and incubated overnight at 4°C with primary antibodies as follows: GLUT1 (Abcam ab-137656) 1:1500, GLUT3 (Santa Cruz Biotechnology Inc B-6; sc-74497) 1:500, SNAT1 (Santa Cruz Biotechnology Inc H-9; sc-137032) 1:2000, SNAT2 (Santa Cruz Biotechnology Inc H-60; sc-67081) 1:500, SNAT4 (Santa Cruz Biotechnology In N-19; sc-3344) 1:1000, FATP1 (Abcam ab-81875) 1:2000, FATP4 (Santa Cruz Biotechnology Inc B-5; sc-10), and tubulin (Sigma T6074) 1:8000. Then, membranes were washed and incubated with the corresponding anti-rabbit, anti-mouse, or anti-goat secondary antibody for each case, using 1:5000 to 1:200000 dilutions during 2 h at 4°C. The membranes were revealed using the Western ECL Substrate and ChemiDoc™ Touch Imaging System (Bio-Rad). Each band of interest was quantified by densitometry using Image-Lab Software 3.0 and normalized to the tubulin expression. Each sample was run in triplicates in different experiments.

Placental Triglyceride Content

Placental triglycerides were quantified by an enzymatic-colorimetric assay. Briefly, 200 mg of the placental tissue was washed in cold PBS, resuspended, and homogenized in 1 ml of 5% NP-40/ddH2O solution using a Polytron PT 1200E homogenizer (Kinematica AG). Samples were heated at 80–100°C in a water bath for 2–5 min or until the NP-40 became cloudy and then cooled down to room temperature. Heating was repeated one more time to solubilize all triglycerides. Samples were centrifuged for 2 min at top speed using a microcentrifuge to remove any insoluble material and diluted 1:5 with ddH2O before proceeding with a commercial triglyceride assay (Spinreact). Results were normalized to the total protein concentration in each sample. (Schwartz and Wolins, 2007).

Maternal Fatty Acid Profile

Maternal serum samples were treated for the quantification of fatty acids according to De La Rocha et al. (2016). Briefly, the sera were lyophilized, and 1 ml of the 0.5 M NaOH solution was added to each sample. Then, 10 μl of the internal standard (methyl pentacosanoate, Sigma-Aldrich) was added at 3 mg/ml, and samples were incubated at 90°C for 1 h in a water bath. The samples were allowed to cool at room temperature for half an hour, then 1.5 ml of boron trifluoride and methanol (Sigma

TABLE 1 | Clinical, biochemical, and anthropometric characteristics of mothers and their SGA, AGA, and LGA newborns.

	SGA (n = 14)	AGA (n = 18)	LGA (n = 10)	LGA-OB (n = 9)	*p*-value
Maternal characteristics					
Age (years)	22 (20–28)	21 (20–29)	27 (23–31)	25 (21–32)	0.250
Pregestational BMI (kg/m^2)	22.7 ± 2.9	23.9 ± 3.2	25.4 ± 2.8^{+}	31.3 ± 1.3^{*+}	**<0.001**
Gestational weight gain (kg)	11.1 ± 4.4	13.5 ± 4.6	13.8 ± 4.5	12.8 ± 2.7	0.412
Blood glucose (mg/dl)	73.6 ± 14.8	82.6 ± 11.1	75.6 ± 13.7	78.8 ± 16.2	0.292
HbA1c (%)	5.2 (4.8-5.9)	5.5 (5.3-5.7)	5.9 (5.4-6.0)	5.6 (4.8-6.1)	0.489
Triglycerides (mg/dl)	177 (155-220)	225 (198-265)	217 (159-302)	215 (173-299)	0.265
Total cholesterol (mg/dl)	192 ± 36	209 ± 30	198 ± 41	210 ± 51	0.541
Insulin (uUI/l)	8.4 (5.0-26.2)	9.7 (6.0-13.9)	19.4 (7.7-30.9)	9.6 (7.3-29.0)	0.412
Neonatal characteristics					
Baby's gender (male/female)	6/8	11/7	7/3	4/5	0.191
Delivery method (vaginal/c-section)	7/7	12/6	6/4	2/7	0.170
Gestational age (wk)	38.4 ± 1.1	38.7 ± 1.1	39.8 ± 0.9^{*+}	38.9 ± 0.6	**0.012**
Placental weight (g)	404.9 ± 64.2 *	608.7 ± 112.0	730.1 ± 80.3^{*+}	740.1 ± 119.8^{*+}	**<0.001**
Birth weight (g)	2,307 (2,143.8-2,407.8)*	3,272.5 (2,925.0-3,336.3)	3,915 (3,787.5-4,102.5) $^{*+}$	3,893 (3,815-4,127.5)$^{*+}$	**<0.001**
Birth lenght (cm)	45.6 ± 2.3*	50.9 ± 2.1	52.8 ± 2.1^{*+}	52.2 ± 2.1^{+}	**<0.001**
Head circumference (cm)	32.2 ± 1.7*	34.6 ± 1.5	36.4 ± 1.6^{*+}	36.3 ± 0.7^{*+}	**<0.001**
Thoracic circumference (cm)	29.7 ± 1.1*	33.6 ± 1.6	35.8 ± 1.2^{*+}	35.2 ± 1.0^{*+}	**<0.001**
Abdominal circumference (cm)	27.3 ± 1.4*	30.8 ± 1.8	34.1 ± 2.2^{*+}	33.9 ± 2.2^{*+}	**<0.001**
Ponderal index (g/cm^3)	2.52 (1.84-2.88)	2.42 (2.23-2.55)	2.71 (2.5-2.88)	2.75 (2.60-3.06) *	**0.004**
Triglycerides (mg/dl)	53.6 (36.3-71.4)	58.4 (54.0-66.6)	49.0 (45.0-58.8)	44.9 (38.0-52.85)	0.090
Total cholesterol (mg/dl)	69.9 (59.8-86.2)	83.7 (75.9-95.4)	76.5 (70.3-79.4)	65.4 (59.5-84.1)	0.053
HDL (mg/dl)	41.02 ± 7.6*	47.6 ± 7.1	42.2 ± 10.5	41.6 ± 6.4	0.087
LDL (mg/dl)	16.2 (11.4-30.8)	25.5 (10.0-34.1)	22.2 (11.7-29.5)	20.8 (11.1-30.1)	0.434
Glucose (mg/dl)	63.7 (45.1-85.1)*	77.1 (66.5-90.2)	61.8 (56.7-70.5)	63.5 (57.5-74.4)	0.07
Insulin (uUI/l)	1.6 (0.8-2.9)*	3.5 (2.4-4.9)	4.1 (3.5-5.3) $^{+}$	4.1 (1.4-7.7)$^{+}$	**0.003**

Mean ± SD; median (25–75% quartile range); *p < *0.05 compared to control AGA,* $^{+}$p< *0.05 compared to SGA.*

Aldrich) were added, and incubated at 90°C for 30 min. Next, 3 ml of hexane was added, and each sample was vortexed for 2 min at medium speed, centrifuged for 5 min at 3,000 rpm at 4°C, and the organic phase was recovered, in clean conical tubes. The samples were stored at 4°C until use.

Before analyzing the samples, the hexane from the organic phase was evaporated to dryness with nitrogen gas, and the pellet was resuspended with 400 µl of isooctane (2,2,4-Trimethylpentane, Sigma-Aldrich) and transferred to a gas chromatography vial.

The samples were analyzed using a gas chromatograph (Perkin Elmer Clarus 680-5Q8), with a capillary column (Elitewax 10: 30 m × 0.25 mm ID 0.24 µm) coupled to a flame ionization detector (GC-FID). The programmed oven temperature ramp was as follows: initial temperature 40°C for 1 min increasing at a rate of 10°C/min up to 150°C, maintaining 1 min, and the final temperature was 230°C increasing at a rate of 5°C/min holding 20 min. The injector temperature was kept constant at 250 °C. Helium was used as the carrier gas at a flow rate of 2 ml/min. The sample was injected at a ratio of 10:1 split. TotalChrom Navigator-Clarus software was used for the data analysis. Fatty acid identification was made by comparing the retention times with the standards (Sigma-Aldrich) of each of the fatty acids of interest (palmitic, arachidonic, linoleic, and docosahexaenoic (DHA) acids) and normalized to the percentage of total fatty acids in the sample.

Statistical Analysis

Data distribution was assessed by the Kolmogorov–Smirnov Test. Clinical data are presented with mean ± SD or median and interquartile range, according to distribution data. Differences between each experimental group were evaluated by ANOVA or Kruskal–Wallis tests. Spearman coefficients were used to evaluate correlations, and the multiple linear regression was performed to confirm associations. *p values* less than 0.05 were considered statistically significant. StatView statistical analysis software V.4.5 (Abacus Concepts, Berkeley, CA, United States) was used.

RESULTS

Anthropometric, Clinical, and Biochemical Data

Anthropometric, clinical, and biochemical data of mothers and newborns are detailed in **Table 1**. The pregestational BMI of mothers from the LGA group was higher than that of mothers of SGA newborns ($p < 0.05$). LGA-OB mothers presented higher pregestational BMI in comparison with mothers of AGA ($p < 0.05$) and SGA newborns ($p < 0.05$). Gestational weight gain was not different between the groups, even after adjusting for weeks of gestation ($p = 0.929$, data not shown). No other differences between groups were observed in the metabolic profile of mothers.

Anthropometric parameters in SGA, LGA, and LGA-OB newborns were different compared with AGA newborns. Birth weight, birth length, head circumference, thoracic circumference, and abdominal circumference were lower in SGA newborns ($p > 0.05$) and higher in LGA and LGA-OB newborns than AGA. Interestingly, only LGA-OB newborns differed in their ponderal index, being higher than the AGA group.

Cord blood HDL cholesterol, glucose, and insulin were 13.8, 17.4, and 54.3% lower in SGA, respectively, compared to AGA. In addition, cord blood insulin of LGA and LGA-OB was 17.1% higher than in SGA newborns. Cord blood triglycerides, total cholesterol, and LDL cholesterol were similar between the groups.

Placental Expression of Glucose Transporters

The protein expression of the placental glucose transporter GLUT1 was higher in LGA and lower in SGA than the AGA group (**Figures 1A,B**). The GLUT1 expression in the LGA-OB group was similar to that of AGA but lower than the LGA group (**Figures 1A,B**). GLUT1 positively correlated with maternal HbA1c, placental weight, and neonatal abdominal perimeter (**Figures 1C, F, and G**, respectively). GLUT1 also correlated with the ponderal index (r = 0.3 and $p = 0.032$). The placental expression of GLUT3 did not differ between groups (**Figures 1D,E**). Both GLUT1 and GLUT3 expressions were similar between male and female newborns ($p = 0.635$ and 0.102, respectively).

Placental Expression of Amino Acid Transporters

The placental expression of amino acid transporters SNAT1, SNAT2, and SNAT4 was evaluated. The SNAT1 expression showed no differences between SGA, AGA, LGA, and LGA-OB (**Figures 2A,B**), or either male or female placentas ($p = 0.323$) and did not correlate with any other maternal or neonatal variables. The protein expression of SNAT 2 was lower in SGA than that in AGA (**Figures 2C,D**). Placental SNAT4 was lower only in LGA-OB than that in AGA and SGA (**Figures 2E,F**). SNAT4 also correlated with placental weight (**Figure 2G**), birth weight (**Figure 2H**), and most neonatal anthropometrical variables such as size at birth (r = −0.289 and $p = 0.04$), thoracic perimeter (r = −0.31 and $p = 0.03$), and abdominal perimeter (r = −0.39 and $p = 0.004$). We found a positive correlation between SNAT1 and SNAT4 protein expressions (r = 0.297 and $p = 0.035$). No differences by neonatal sex were found for these transporters ($p = 0.422$ for SNAT2 and $p = 0.084$ for SNAT4).

Placental Expression of Fatty Acid Transporters

The fatty acid transporter FATP1 was lower in SGA placentas than in LGA and LGA-OB groups, while only LGA was higher than AGA (**Figures 3A,B**). The FATP1 expression positively correlated with placental weight (**Figure 3C**) and birth anthropometry, including birth weight (**Figure 3F**), size at birth (r = 0.436 and $p = 0.001$), cephalic perimeter (r = 0.28 and $p = 0.05$), thoracic perimeter (r = 0.58 and $p = 0.006$), and abdominal perimeter (r = 0.442 and $p = 0.001$). The placental FAPT4 expression was not different according to birth weight groups (**Figures 3D,E**). Placentas from male and female newborns showed similar FATP1 and FATP4 expressions ($p = 0.656$ and 0.528, respectively).

Placental Triglyceride Content

We assessed the placental triglyceride content between the different birthweight groups. Placental triglycerides were elevated both in LGA and LGA-OB compared to AGA and SGA (**Figure 4A**), with no differences between sexes. The triglyceride content correlated positively with pregestational BMI, maternal insulin (**Figures 4C,D**), and maternal HOMA-IR (r = −0.302 and $p = 0.033$), together with birth weight (**Figure 4B**), size at birth (r = 0.32 and $p = 0.021$), cephalic perimeter (r = 0.33 and $p = 0.02$), thoracic perimeter (r = 0.37 and $p = 0.007$), and abdominal perimeter (r = 0.35 and $p = 0.01$) of newborns.

Maternal Fatty Acid Profile

The percentages of maternal palmitic (PA), linoleic (LA), and arachidonic acids (AA) were no different from birth weight classification, while DHA in mothers from SGA newborns was higher than that in AGA and the other groups (**Figure 5A**), with this difference due to a higher DHA concentration in mothers from males than in females (7.62 ± 0.69 and 4.43 ± 0.77, respectively, $p = 0.004$). Maternal DHA did not differ between males and females in AGA, LGA, or LGA-OB, nor were other differences between sexes found for the rest of the fatty acids (data not shown).

We found a negative correlation between maternal DHA and placental weight, maternal serum triglycerides, cord blood glucose (**Figures 2B–D**), and neonatal abdominal perimeter (r = −0.353 and $p = 0.02$). Although no differences in the other fatty acids were observed, palmitic acid correlated negatively with placental FATP4 (r = −0.384 and $p = 0.01$) and positively with cord blood insulin (r = 0.314 and $p = 0.04$). Linoleic acid correlated with palmitic acid (r = 0.488 and $p = 0.001$) and maternal cholesterol (r = −0.36 and $p = 0.02$). Arachidonic acid was inversely correlated with maternal triglycerides (r = −0.38 and $p = 0.026$) and directly with the FATP4 expression in the placenta (r = 0.34 and $p = 0.045$).

When performing a multivariate analysis, maternal DHA remained significantly associated with birth weight (t = −2.497, $p = 0.018$) and placental weight (t = −2.245, $p = 0.032$), independently of pregestational BMI, pregnancy weight gain, neonatal sex, and gestational age.

DISCUSSION

Adverse factors in early life result in fetal metabolic programming and increased risk of adult diseases, being birth weight an indirect marker of this unfavorable environment. The placenta plays a crucial role in modulating fetal growth not only by transporting nutrients but also by adapting its metabolism and function in

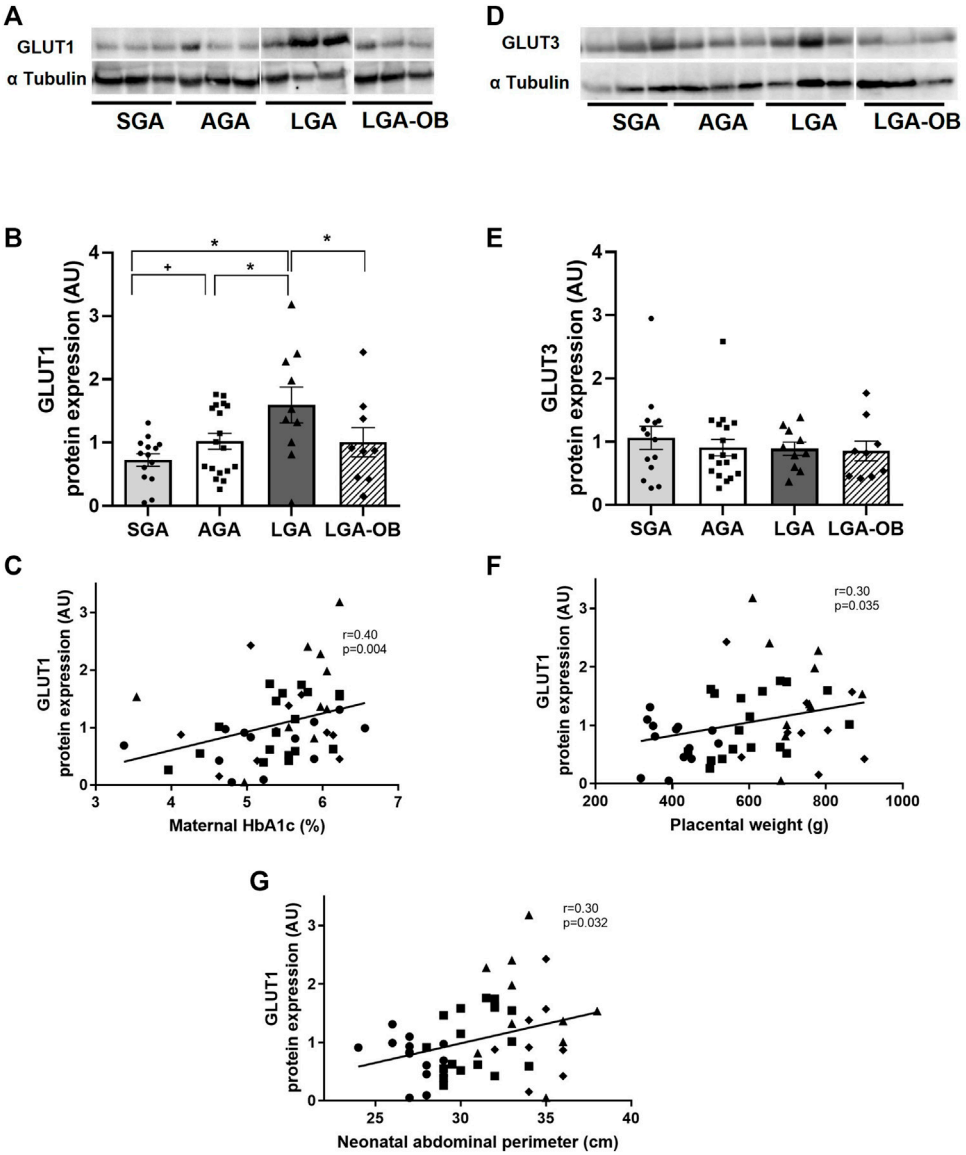

FIGURE 1 | Protein expression of glucose transporters in placentas of SGA, AGA, LGA, and LGA-OB newborns. Representative Western blot of GLUT1 **(A)** and GLUT3 **(D)** in placentas of SGA (n = 14), AGA (n = 18), LGA (n = 10), and LGA-OB (n = 9) newborns. Bands presented correspond to the same membrane. Quantification of GLUT1 **(B)** and GLUT3 **(E)** protein expressions in the placenta by immunodetection. Data are mean ± SE. * denotes significant difference ($p < 0.05$) between groups by ANOVA. + denotes significant difference when compared to control AGA by Student´s *t* test. Correlation between the placental expression of GLUT1 with maternal HbA1c **(C)**, placental weight **(F),** and neonatal abdominal perimeter **(G)**.

response to maternal nutrition throughout pregnancy (James-Allan et al., 2020), thus acting as a nutrient sensor (Jansson and Powell, 2006). Most studies about placental nutrient transport are conducted in the context of pregnancy pathologies, but information on healthy or obese women considering neonatal outcomes, such as birth weight and metabolism, is lacking. In the present study, we evaluated the expression of several placental nutrient transporters in idiopathic birth weight alterations (SGA, AGA, and LGA) and maternal obesity (LGA-OB) in relationship to the maternal metabolic status and neonatal outcomes.

Glucose is the main energy substrate for fetal and placental growth. The fetus depends on maternal glucose availability, which is transported by placental GLUTs (Joshi et al., 2021). GLUT1 (Illsley, 2000) is the main placental glucose transporter, and although it is located primarily in the microvillous membrane (MVM), facing the maternal side of the placenta, the GLUT1 expression in the basal membrane (BM) facing the fetal capillaries is suggested as the rate limiting step for fetal glucose transfer (Jansson et al., 1993; James-Allan et al., 2019).

In the present study, an increased protein expression of GLUT1 was found in term placentas from LGA babies, but

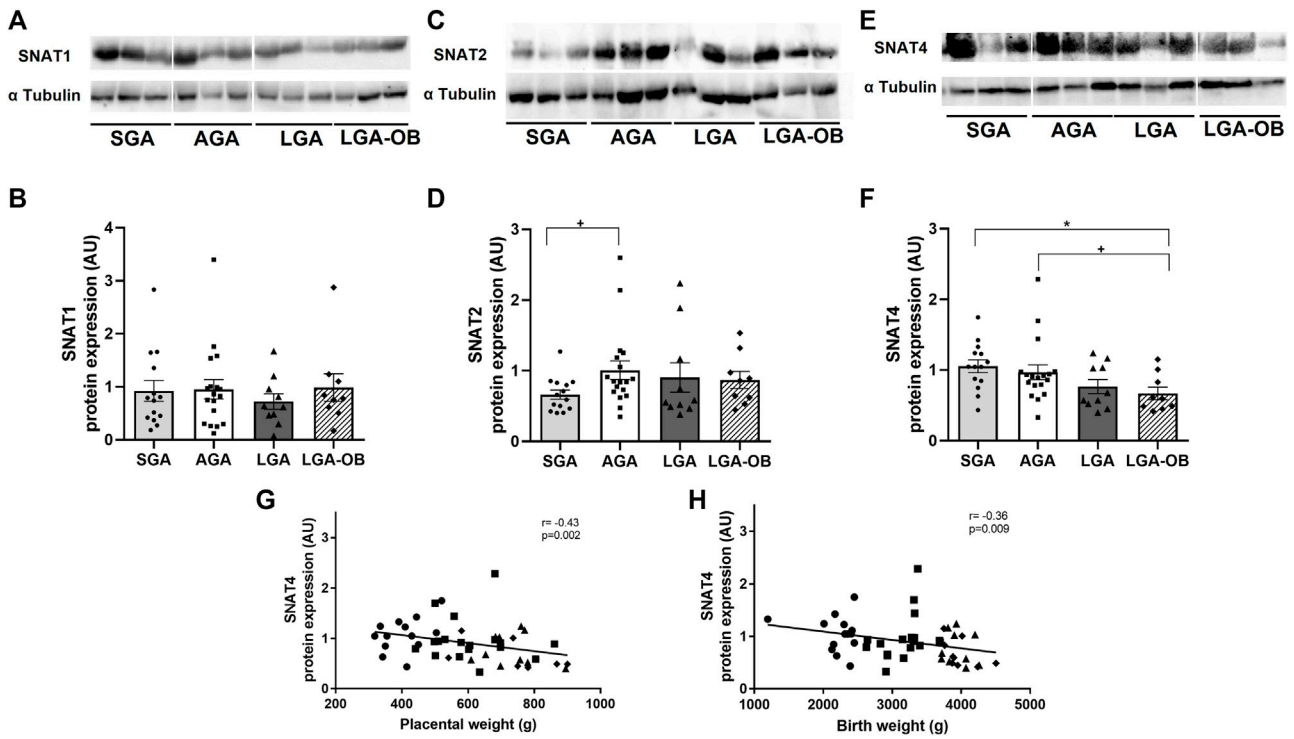

FIGURE 2 | Protein expression of amino acid transporters in placentas of SGA, AGA, LGA, and LGA-OB newborns. Representative Western blot of SNAT1 **(A),** SNAT2 **(C),** and SNAT4 **(E)** in placentas of SGA (n = 14), AGA (n = 18), LGA (n = 10), and LGA-OB (n = 9) newborns. Bands presented correspond to the same membrane. Quantification of SNAT1 **(B),** SNAT2 **(D),** and SNAT4 **(F)** protein expressions in the placenta by immunodetection. Data are mean ± SE. * denotes significant difference ($p < 0.05$) between groups by ANOVA. + denotes significant difference when compared to control AGA by Student´s *t* test. Correlation between the placental expression of SNAT4 with placental weight **(G)** and birth weight **(H)**.

not in LGA-OB, compared to AGA. The overexpression of the GLUT1 transporter has been reported in placentas from mothers with type I diabetes (Jansson et al., 1999) and gestational diabetes [reviewed in Joshi et al. (2021)] although other studies have rendered discordant results (Jansson et al., 2001). Similarly, studies in placentas from women with obesity giving birth to normal weight babies have found no changes in the GLUT1 expression. However, a lower expression in the MVM and a higher expression in the BM have been observed from women with obesity delivering macrosomic newborns (James-Allan et al., 2019), which is consistent with the lack of changes we see in LGA-OB placental samples, comprising both maternal and fetal sides. Few studies have measured GLUT1 in the context of fetal growth restriction, and most of them report no alteration in GLUT1 (Joshi et al., 2021). In a recent study, a decrease of GLUT1 has been showed in IUGR, but not in SGA or macrosomic placentas, contrary to our findings (Stanirowski et al., 2021). These discrepancies may be explained by methodological reasons (immunohistochemistry vs. Western blot).

Several investigations suggest that regulation of the GLUT1 expression occurs through metabolic control and maternal glucose concentrations (Jansson et al., 1999; Joshi et al., 2021). Although the maternal metabolic status did not differ between groups, we found a positive correlation of the GLUT1 expression with maternal HbA1c, suggesting a possible role of the maternal glycemic status in GLUT1 regulation, even in the absence of diabetes mellitus.

It has been widely suggested that the placental GLUTs play a pivotal role in birth weight establishment and that their upregulation may lead to an increase in the glucose transport and fetal growth (Stanirowski et al., 2018; Joshi et al., 2021). The positive correlations between GLUT1 with the placental weight, ponderal index, and neonatal abdominal perimeter found in our study support this notion. Moreover, the altered placental GLUT1 expression is thought not only to regulate the glucose transfer but also to modulate the placental metabolic flexibility. Recently, studies in placental explants have shown that placental glucose dependency is positively correlated with the GLUT1 expression, while glucose flexibility is negatively associated with this transporter, but only in placentas from males (Wang et al., 2019). In contrast, our study found no differences in the GLUT1 expression between male and female placentas.

In opposition to the GLUT1 expression, no differences were found in the expression of GLUT3 in the placentas of the groups evaluated in the present study. While the syncytiotrophoblast GLUT3 expression both in the MVM and BM has been reported in humans (James-Allan et al., 2020), its expression and role in the placenta are less clear (Joshi et al., 2021). Previous studies refer no changes in the GLUT3 expression in gestational diabetes, IUGR, macrosomia (Kainulainen et al., 1997), or maternal obesity

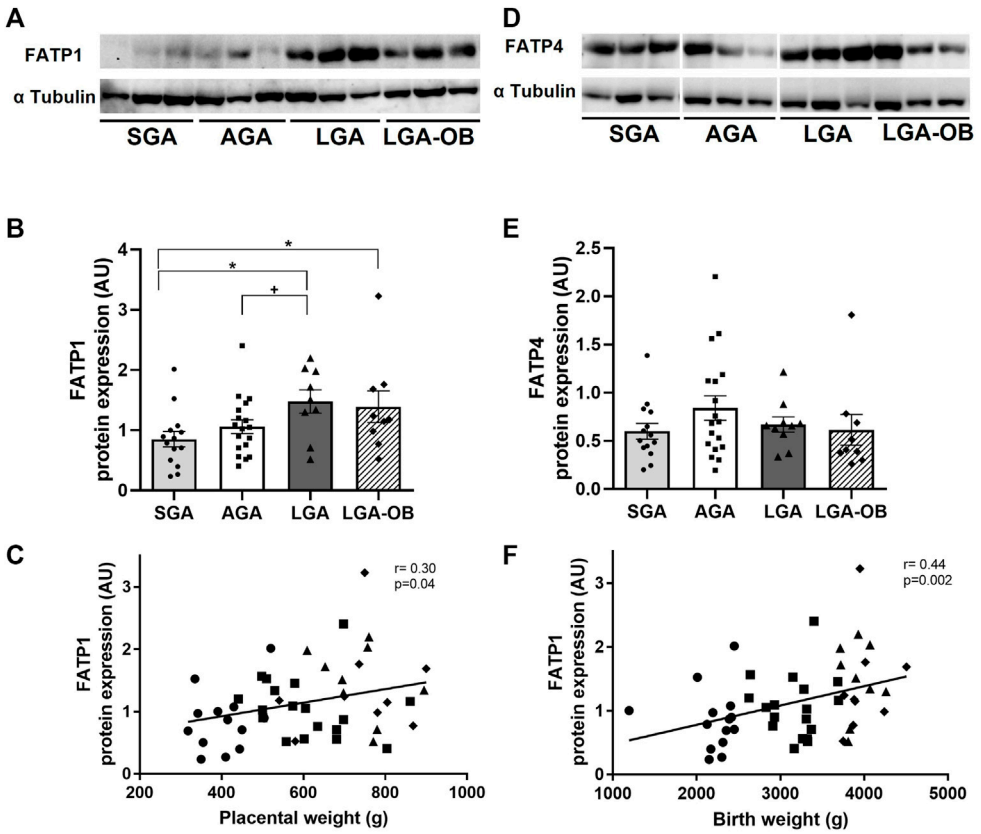

FIGURE 3 | Protein expression of fatty acid transporters in placentas of SGA, AGA, LGA, and LGA-OB newborns. Representative Western blot of FATP1 **(A)** and FATP4 **(D)** in placentas of SGA (n = 14), AGA (n = 18), LGA (n = 10), and LGA-OB (n = 9) newborns. Bands presented correspond to the same membrane. Quantification of FATP1 **(B)** and FATP4 **(E)** protein expressions in the placenta by immunodetection. Data are mean ± SE. * denotes significant difference ($p < 0.05$) between groups by ANOVA. + denotes significant difference when compared to control AGA by Student's *t* test. Correlation between the placental expression of FATP1 with placental weight **(C)** and birth weight **(F)**.

(James-Allan et al., 2020), while an increased expression in IUGR (Janzen et al., 2013; Stanirowski et al., 2021) and decreased expression in gestational diabetes (Zhang et al., 2016) but no changes in SGA (Stanirowski et al., 2021) have been reported in other studies. It is suggested that GLUT3's function in the villous vessels at the maternal–fetal interface is the reuptake of glucose from the fetal circulation, increasing the glucose concentration in the placenta, possibly acting as an adaptive mechanism to protect the fetus from high glucose concentrations (Stanirowski et al., 2018). In this view, the lack of differences or correlation of the GLUT3 expression with maternal or neonatal variables in our study would be expected in the context of healthy normoglycemic mothers.

Placental amino acid transfer to the fetus is mediated by two coordinated systems, termed system A and L. System A transports nonessential neutral amino acids by a sodium-dependent mechanism, comprising the protein transporters SNAT1, SNAT2, and SNAT4, which are mainly located in the MVM (Vaughan et al., 2017). Studies in SNAT2- (Vaughan et al., 2021) or SNAT4 (Matoba et al., 2019)-knockout mice specifically in trophoblasts have shown that these two transporters are crucial for placental and fetal growth, while reduced placental amino acid transfer precedes the onset of IUGR in rat (Pantham et al., 2016) and primate (Rosario et al., 2021) models of maternal protein restriction.

In the present study, the SNAT1 expression showed no differences between birthweight groups. However, SNAT2 was lower in placentas from SGA newborns. These results resemble the findings in primate models of maternal nutrient restriction, in which a decrease in the capacity and activity of system A transport and in birth weight is found, together with a decreased placental expression of SNAT2, but normal SNAT1 (Kavitha et al., 2013). Also, protein (Rosario et al., 2011) or folate (Rosario et al., 2017) restriction during pregnancy decreases the expression of SNAT2 in mice. A study in human SGA from healthy mothers found a reduction in total placental system A amino acid transport activity, without measuring the SNAT expression (Shibata et al., 2008). In the human placenta from preterm and term IURG, lower SNAT1 (Chen et al., 2015) and SNAT2 (Mandò et al., 2013; Chen et al., 2015) protein expressions have been found. In the present study, the SNAT2 expression was similar between AGA, LGA, and LGA-OB, and no correlation of this transporter with birthweight was found. Conversely, the system A activity has been found increased in

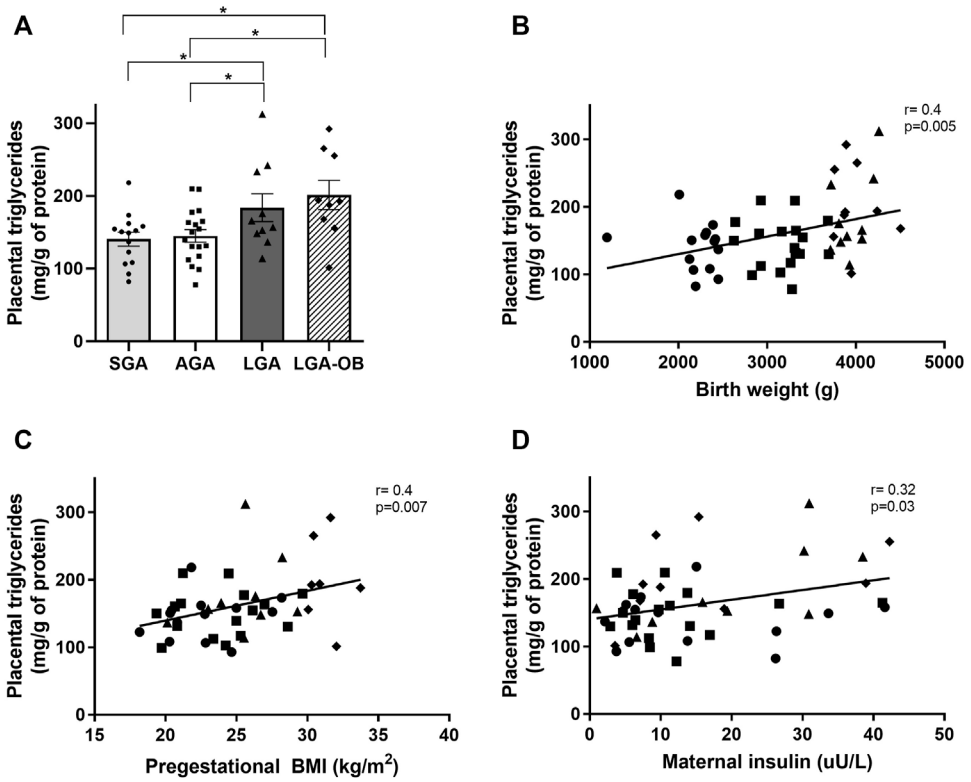

FIGURE 4 | Triglyceride content in placentas of SGA, AGA, LGA, and LGA-OB newborns and correlation with neonatal and maternal variables. **(A)** Quantification of triglycerides in placentas of SGA (n = 14), AGA (n = 18), LGA (n = 10), and LGA-OB (n = 9) newborns by enzymatic-colorimetric method. Results were normalized to total protein in each sample. * denotes significant difference ($p < 0.05$) between groups by ANOVA. Correlation between placental triglycerides with birth weight **(B)**, pregestational BMI **(C),** and maternal insulin **(D)**.

placentas from large babies of obese women, being SNAT2, but not other isoforms, correlated with birth weight and pregestational BMI, although the comparison between normal and obese BMI or AGA and LGA babies was not reported (Jansson et al., 2013).

The placental expression of SNAT4 was lower only in placentas of the LGA-OB group compared to AGA and SGA. Furthermore, a negative correlation was observed between this transporter and placental weight and birth anthropometry. The placental amino acid transport decreases in placentas of obese women with normal weight babies, potentially associated with maternal leptin resistance. Also, immunohistochemistry analysis has revealed a decrease in SNAT4, but not other isoforms, in these placentas (Farley et al., 2010).

Studies exploring the contribution of each SNAT isoform to amino acid transport in the normal human placenta have proposed that SNAT1 is the main mediator of α-methylaminoisobutyricacid (MeAIB) uptake in the MVM, accounting for approximately 70% of the transport, followed by 30% SNAT4 and 1% SNAT2 in humans. Nevertheless, the regulation of the expression of amino acid transporters could be mediated according to its specific substrate availability (Takahashi et al., 2017). SNAT4 has low affinity for MeAIB and greater affinity to cationic amino acids (arginine and lysine) (Hatanaka et al., 2001). The placenta increases system A activity depending on the type and concentrations of amino acids available in order to ensure the optimal fetal growth (Snook Parrott et al., 2007). Hence, the placental SNAT4 expression may serve as a compensatory mechanism, either due to a decrease (Belkacemi et al., 2011) or an increase in nutrient availability (Farley et al., 2010), or else, a saturation of SNAT1 and SNAT2 transporters (Takahashi et al., 2017), which is supported by the positive correlation between SNAT1 and SNAT4 found, and a lower SNAT4 expression in the placenta of obese mothers.

The role of FATPs in the human placenta and their regulation is poorly understood. In the present study, FATP1, but not FATP4, placental expression was positively correlated with placental weight and birth anthropometry, being lower in SGA and higher in both LGA and LGA-OB. Similarly, FATP1 mRNA has been correlated with maternal BMI in human placentas (Hirschmugl et al., 2017). On the contrary, FATP1 mRNA has found to be lower in placentas from women with overweight or obesity, together with high placental long-chain polyunsaturated fatty acids (LCPUFAs) and low saturated fatty acids (Segura et al., 2017). FATP1 transports long-chain fatty acids, and its gene expression in the placenta has been positively correlated with placental DHA in placental phospholipids and triglycerides and negatively with cord blood AA in phospholipids (Larqué et al.,

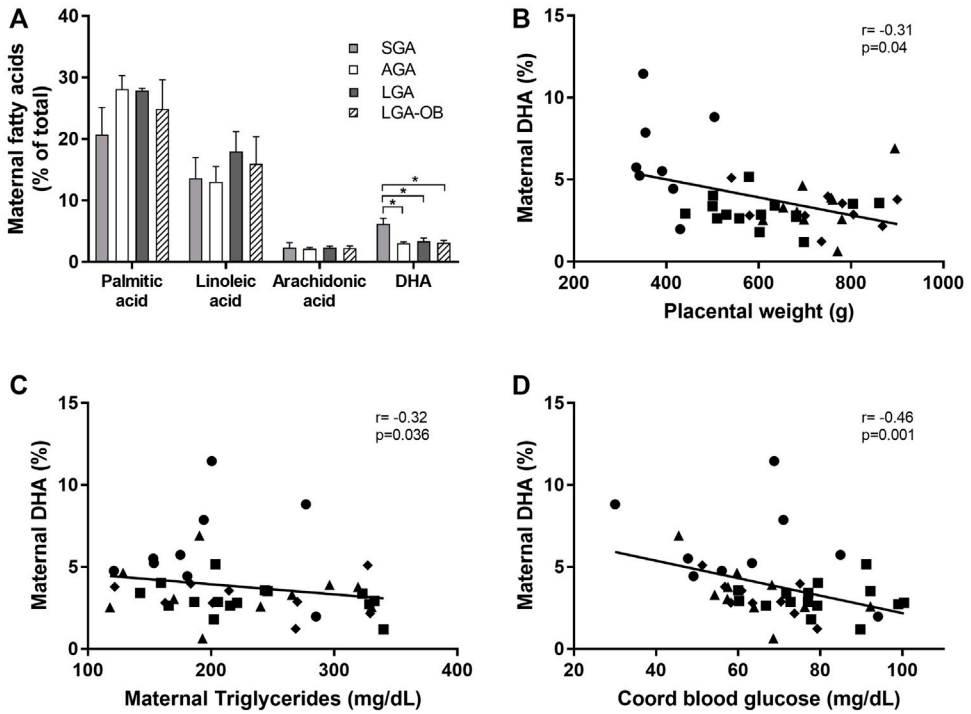

FIGURE 5 | Maternal serum fatty acid profile and correlation of maternal DHA with maternal and neonatal variables. Maternal serum fatty acids **(A)** evaluated by gas chromatography. Fatty acid identification was made by comparing the retention times with the standards of each of the fatty acids of interest (palmitic, arachidonic, linoleic, and DHA acids) and normalized to the percentage of total fatty acids in the sample. * denotes significant difference ($p < 0.05$) between groups by ANOVA. Correlation between maternal DHA with placental weight **(B)**, maternal triglycerides **(C),** and cord blood glucose **(D)**.

2006). Moreover, the FATP1 expression is also associated with a greater uptake of palmitate and increased triglyceride accumulation when overexpressed in myotube C2C12 cells (Kawaguchi et al., 2014). Also, an increase in FATP1 mRNA has been show in cultured human trophoblast cells with PPARg and RXR agonists, suggesting that regulation of this transporter may also be dependent on the different types and concentrations of maternal circulating nutrients (Schaiff et al., 2005). Supporting this view, other nutrients have been associated with the FATP1 expression, such as vitamin B12, whose restriction in mice lowers its mRNA, possibly by epigenetic mechanisms (Wadhwani et al., 2013).

To further explore if changes in FATP1 protein could be related to placental lipid accumulation or nutrient availability, we measured total placental triglycerides (TG) and plasmatic fatty acid maternal profiles. The placental triglyceride content was higher in LGA and LGA-OB, similar to previous reports in women with obesity (Hirschmugl et al., 2017) but in contrast to other studies (Hulme et al., 2019), possibly due to the fact that birth weight groups were not taken into account in previous studies. Placental TG correlated positively with birth anthropometry and maternal pregestational BMI. Moreover, a correlation with maternal insulin and HOMA-IR was found, but not with FATP1 or FATP4, which is consistent with *in vitro* studies in the human placenta, where exposure to elevated glucose alone (Hulme et al., 2019) or in combination with high insulin exposure (Mishra et al., 2020) promotes placental TG accumulation without changing the FATP expression.

Maternal LCPUFAs, such as DHA and AA, are determinant for fetal growth and, particularly, for fetal brain development and postnatal neurodevelopment, as well as for the placental metabolism itself (Duttaroy and Basak, 2020). In the present study, total maternal DHA was negatively associated with birth weight and abdominal perimeter independently from other maternal factors, presumably through an effect of DHA on the placental weight or function. These results agree with a recent study, in which concentrations of maternal and umbilical DHA in different phospholipid subclasses were lower in obese mothers of male fetuses, but not females, despite an increase in the lysophosphatidylcholine-DHA transporter MFSD2a in BM from male placentas, suggesting an adaptative mechanism of DHA transport. Similarly, no changes in FATPs in either the MVM or BM were found (Powell et al., 2021). Our findings further expand these observations regarding the relevance of sexual dimorphism for placental function, particularly in lipid transfer and metabolism, as an increase in total maternal DHA in SGA, attributable to mothers from SGA males, was found, just as previously shown for maternal obesity (Powell et al., 2021). Indeed, an increasing body of evidence has demonstrated that placentas from females grow slower than placentas from males, and both differ in their adaptative responses to environmental factors (Kalisch-Smith et al., 2017), which emphasizes the

importance of evaluating males and females separately in placenta studies.

One of the main strengths of the present study is the inclusion of placentas from both extremes of birth weight, allowing to compare these two opposite conditions in healthy pregnancy, which may pose an increased risk for those newborns in metabolic disease in the future life. In particular, by separating the LGA newborns by the presence or absence of pregestational obesity, differences in the nutrient transporter GLUT1, but not in amino acid or fatty acid transporters, could be detected. Moreover, despite a similar metabolic profile and ponderal weight gain compared to AGA newborns, both LGA and LGA-OB mothers present hyperinsulinemia, while the LGA from obese mothers may have increased adiposity, which is suggested by the higher ponderal index found only in this group. Together with our findings in placental TG and maternal fatty acids, we can conclude that LGA babies born from normal or obese mothers have altered placental adaptations, which can possibly be exacerbated by maternal adiposity and hormonal status.

The lack of precise nutritional information is indeed a limitation to the study. It has been described that a diet of high fat and sugar induces alterations in the expression and activity of placental nutritional transporters, which has negative consequences on the growth of the offspring (Rosario et al., 2015). Having this information would surely help in elucidating the mechanisms underlying the differences between the study groups, mostly for the fatty acid profile, but also for those transporters whose placental expression has been associated with maternal macro or micronutrients, such as SNAT2 (Matoba et al., 2019) (51) and FATP1 (Schaiff et al., 2005; Wadhwani et al., 2013).

Another limitation is that the transporter expression was measured at term in placental samples including both the MVM and BM together, and their activity was not evaluated. Thus, we cannot establish if the differences in the protein expression could account for modifications in glucose, amino acid, or fatty acid transport. Indeed, the protein expressions of SNAT2, SNAT4, and GLUT1 are higher in term placentas compared to early gestation ones, and FATP4 is lower. Also, system A amino acid transport is higher at term (James-Allan et al., 2020). Moreover, location of the transporters at the membrane also influences their activity. Particularly, downregulation of mTORC1 has been shown to modulate the ubiquitination of SNAT2 by increasing ubiquitin ligase Nedd4-2, which favors the proteosomal degradation of the protein, thus decreasing its expression in the plasma membrane (Chen et al., 2015; Rosario et al., 2016). In a recent study, we found that the mTOR expression and activation was positively associated with the placental weight and birth weight, while AMPK, an upstream kinase-inhibiting mTOR, correlated negatively with the birth weight in healthy mothers (Lazo-de-la-Vega-Monroy et al., 2020). Together, these results support the potential role of nutrient-sensing pathways in modulating the expression and activity placental transporters and thus fetal growth. More studies in normal and pathological human pregnancies integrating the expression, location, and activity of transporters' isoforms and their regulation, either by hormonal or nutritional factors, will increase the knowledge in the mechanisms modulating the placental nutrient transport.

In conclusion, we found that the placental protein expression of specific nutrient transporters is related to placental weight establishment and fetal growth and may depend on maternal factors such as glycemia and pregestational BMI even in the absence of pregnancy disorders. Additionally, we reported differences in the placental TG content and maternal DHA status associated with birth weight, with sexual dimorphism in circulating maternal DHA of SGA mothers. Further studies evaluating the potential mechanisms for regulating the expression of these nutrient transporters, such as maternal micronutrients or placental energy sensors, will open the door for potential therapeutic targets, as well as for defining the quality and quantity of nutrients required for placental function and hence fetal growth and future metabolic diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committees of the University of Guanajuato and the corresponding health institutions (HGL-GTSSA002101-337 and CIBIUG P-23-2015 y P-43-2017). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

M-LL-V-M and GB-S conceived and designed the study. M-LL-V-M, J-AG-S, G-CR-S, M-AS-B, M-IG-D and M-AC-F generated, collected, and assembled the data. J-AG-S, M-LL-V-M, GB-S, H-MG-Z and J-MM analyzed and interpreted the data, drafted, and critically revised the manuscript. All authors approved the final version to be published.

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Hepatic Epigenetic Reprogramming After Liver Resection in Offspring Alleviates the Effects of Maternal Obesity

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Obesity has become a public health problem in recent decades, and during pregnancy, it can lead to an increased risk of gestational complications and permanent changes in the offspring resulting from a process known as metabolic programming. The offspring of obese dams are at increased risk of developing non-alcoholic fatty liver disease (NAFLD), even in the absence of high-fat diet consumption. NAFLD is a chronic fatty liver disease that can progress to extremely severe conditions that require surgical intervention with the removal of the injured tissue. Liver regeneration is necessary to preserve organ function. A range of pathways is activated in the liver regeneration process, including the Hippo, TGFβ, and AMPK signaling pathways that are under epigenetic control. We investigated whether microRNA modulation in the liver of the offspring of obese dams would impact gene expression of Hippo, TGFβ, and AMPK pathways and tissue regeneration after partial hepatectomy (PHx). Female Swiss mice fed a standard chow or a high-fat diet (HFD) before and during pregnancy and lactation were mated with male control mice. The offspring from control (CT-O) and obese (HF-O) dams weaned to standard chow diet until day 56 were submitted to PHx surgery. Prior to the surgery, HF-O presented alterations in miR-122, miR-370, and Let-7a expression in the liver compared to CT-O, as previously shown, as well as in its target genes involved in liver regeneration. However, after the PHx (4 h or 48 h post-surgery), differences in gene expression between CT-O and HF-O were suppressed, as well as in microRNA expression in the liver. Furthermore, both CT-O and HF-O presented a similar regenerative capacity of the liver within 48 h after PHx. Our results suggest that survival and regenerative mechanisms induced by the partial hepatectomy may overcome the epigenetic changes in the liver of offspring programmed by maternal obesity.

Keywords: partial hepatectomy, microRNAs, metabolic programming, DOHaD, obesity, liver regeneration, NAFLD

INTRODUCTION

Obesity is a global epidemic as its prevalence has more than tripled since 1975 (World Health Organization, 2018). Excessive fat accumulation is considered a major risk factor for several chronic diseases, such as non-alcoholic fatty liver disease (NAFLD) (Younossi et al., 2016). NAFLD is defined as an ectopic lipid accumulation within the hepatocytes up to 5% in the absence of drugs or alcohol consumption (Lindenmeyer and McCullough, 2018), which can progress to stages with associated inflammation and fibrosis, fatally causing liver failure (Koppe, 2014; Bellentani and Med Stefano Bellentani, 2017). Recent studies have demonstrated that NAFLD development can be triggered during fetal and early stages of life, as a consequence of exposure to a maternal obesogenic environment, such as a high-fat diet (HFD) consumption (Hoover et al., 1987; Fernandez-Twinn et al., 2015; Simino et al., 2021). In addition, the HFD consumption by dams predisposes offspring to higher body weight and adiposity, dyslipidemia, and insulin resistance at different stages of life (Melo et al., 2014; Fante et al., 2016; Lemes et al., 2018; Castro-Rodríguez et al., 2021). The development of these metabolic abnormalities in the offspring, as well as NAFLD, has shown to be under epigenetic control (Benatti et al., 2014; Fernandez-Twinn et al., 2015; Bianco-Miotto et al., 2017; Simino et al., 2021).

Recently, microRNA (miRNA) modulation has been associated with offspring outcomes programmed by maternal obesity during gestation and/or lactation (Benatti et al., 2014; Simino et al., 2017; Sun et al., 2020; Simino et al., 2021). miRNAs are small molecules of non-coding RNAs that mostly act as a target mRNA silencer (Correia de Sousa et al., 2019). Weanling and adult offspring of obese dams have altered hepatic miRNAs that impact the key lipid enzymes causing fat liver accumulation (Benatti et al., 2014; Simino et al., 2017). We have shown that mice offspring programmed by maternal obesity exhibit higher levels of miR-370 and Let-7 that target *Cpt1a* and *Prkaa2* (AMPKα2 encoding gene), respectively, possibly resulting in lower fat oxidation in the liver (Simino et al., 2017; Simino et al., 2021). In addition, the offspring of maternal obesity have reduced levels of hepatic miR-122, which is the main miRNA in the liver that targets key genes involved in triglyceride synthesis, such as *Agpat*, thus promoting lipogenesis (Simino et al., 2017). As a result of this, the offspring of obese dams display an ectopic fat liver accumulation and become predisposed to an increased risk of metabolic syndrome development.

The progression of NAFLD to more severe conditions may require surgical intervention, such as bariatric surgery, liver transplantation, or partial hepatectomy (PHx), the latter involving the removal of the injured tissue to preserve the organ functions (Koppe, 2014; Shingina et al., 2019; Lassailly et al., 2020). The experimental studies have shown that obese mice with NAFLD have a major incidence of complications and death after liver resection surgery or liver transplantation (Todo et al., 1989; Behrns et al., 1998; Murata et al., 2007; Jin et al., 2019; Kumar et al., 2019). Human epidemiological evidence has demonstrated that obese patients have lower liver regeneration index and kinetic growth rate of the liver after major hepatic resection than eutrophic patients (Truant et al., 2013; Amini et al., 2016).

Liver resection through PHx promotes great cellular stress, inducing inflammatory cytokine production and release which signal the initial phase of the liver regeneration with the G1 cell cycle, and subsequent activation of several genes involved in cell replication (Li et al., 2001; Mao et al., 2014). We have recently shown that mice offspring programmed by maternal obesity during gestation and lactation have delayed regenerative response after PHx since they present lower IL-6 levels, Ki67 labeling, cells in S-phase, and Cyclin D1 content as compared to the offspring of control dams. However, even when rechallenged to HFD in adult life, PHx had lower impact on the survival rate of the offspring of obese dams, since they reach successful liver regeneration as the offspring of control dams (Fante et al., 2021).

Although liver regeneration processes have been largely studied, little is known about the influence of hepatic miRNA modulation on liver regeneration after partial hepatectomy in offspring programmed by maternal obesity. We investigated whether the hepatic miRNA alterations previously reported in offspring programmed by maternal obesity would impact key gene expression that could affect the tissue regeneration processes after PHx.

METHODS

Experimental Design

Female and male Swiss mice (5 weeks old, $n = 10$ females per group) were obtained from the Animal Breeding Center at the University of Campinas (CEMIB/Unicamp, Brazil). The experiments were approved by the Committee for Ethics in Animal Experimentation (protocol #4594-1/2017) at the State University of Campinas–UNICAMP and were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications #8023, revised 1978). The females were randomly separated into two groups: the standard chow-fed group (CT, NUVILAB® Cr-1; Nuvital; 3.5 kcal/g, 9.5% of energy from fat) and a high-fat diet-fed group (HF for growth; AIN-93G; 4.6 kcal/g, 45% of energy from fat) both *ad libitum* during the adaptation period (4 weeks before mating), gestation, and lactation. The HF diet was prepared according to previous studies (Simino et al., 2017), and only obesity-prone females were included in the HF group (80% belonging to the cohort described by Fante et al., 2021). All mice had free access to water and were maintained in individual polypropylene micro-isolators at 22 ± 1°C with lights on from 06:00 to 18:00 h. Swiss males from the same age fed CT diet were used for mating (2 females:1 male). After birth, both litters of control dams (CT-O) and HF dams (HF-O) were adjusted to eight male pups per dam, and females were discarded. When the number of males was not enough, females were kept until weaning. After weaning, the offspring were fed CT diet *ad libitum* until 8 weeks of age (p56), when they were submitted to a PHx procedure. Euthanasia was undertaken in all mice after intraperitoneal anesthesia (ketamine: xylazine: diazepam ratio 3:2:2) followed decapitation. One male pup of each litter was used for analysis.

Partial Hepatectomy

After inhalation anesthesia with isoflurane and oxygen (2%:2L/min), a PHx procedure was performed at p56 to remove ⅔ of the liver and assess the hepatic regenerative capacity of offspring

(Mitchell and Willenbring, 2008). After surgery, the animals received analgesia every 24 h until the sacrifice (carprofen-10 mg/kg of body weight). The left lobe was removed during surgery and used for time 0 h analyses (baseline), and the right lobe was used for analyses at 4 and 48 h after PHx. To avoid discrepancies caused by methodological artifacts, all the surgeries were conducted by the same researcher and the liver samples with hemorrhage resulting from stenosis of the suprahepatic vena cava were excluded, as stated by Mitchell and Willenbring (2008).

Body Composition and Serum Biochemical Analysis

The following analyses were performed in dams and offspring from both groups. Bodyweight was measured weekly, and adiposity was evaluated by the ratio of epididymal white adipose tissue mass, collected after the euthanasia, and total body weight. Fasting glucose was assessed using the Accu-Chek Performa glucometer (Roche®, Switzerland; detection limit: 10 mg/dl). Triglycerides (TG) and cholesterol (CHOL) serum content were quantified using enzymatic kits (Laborlab, Brazil; detection limit: 0.8–3.0 mg/dl), according to the manufacturer's guidelines. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using a kinetic spectrophotometric method in offspring serum in the baseline and after 4 and 48 h of PHx according to the manufacturer's guidelines (ALT: Labtest ref.: 108; AST: Labtest ref.: 109, Brazil; detection limit: 1.75U/L). Serum insulin was determined using the Rat/Mouse Insulin ELISA Kit (Millipore, Germany; 1.3–4.0 ng/ml sensitivity).

In Silico Analysis of miRNA Potential Targets

In silico analyses were performed to identify predicted targets genes involved in cell cycle, growth, and proliferation in *Mus musculus* considering those that match with miRNAs previously described as altered in the offspring of obese dams (miR-370, Let-7, and miR-122) (Simino et al., 2017; Simino et al., 2021). The miRNA/mRNA target prediction was performed using the MiRWalk 2.0 platform* accessing a total of 12 algorithms. Interactions were considered valid when predicted by TargetScan algorithm and, at least, five other algorithms. Then, the targets were included in the DAVID platform** to select target genes involved in cell cycle and proliferation.

Real-Time Polymerase Chain Reaction After Reverse Transcription (RT-qPCR)

The total RNA and miRNA were extracted from the liver or cells using RNAzol reagent (Sigma Aldrich) according to the manufacturer's recommendations and quantified using NanoDrop ND-2000. Reverse transcription was performed with 3 µg of total RNA or miRNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The relative expression of mRNAs (Smad3 ID Mm01170760_m1, Nf2 ID Mm00477771_m1, Yap1 Mm01143263_m1, Il6 ID Mm00446190_m1, Il1b ID Mm00434228_m1, Tnf ID Mm00443258_m1, Prkaa2 ID Mm01264789_m1, and Actb ID 4352341E) and miRNAs (miR-122 ID 002245, miR-370 ID 002275, Let-7a ID 000377, and U6srRNA ID 001973) was determined using a Taqman system. qPCR was performed with 20 ng of complementary DNA on the ABI 7500 Fast System. Data were expressed as relative values determined by the comparative threshold cycle method (2-ΔΔCt), according to the manufacturer's recommendation.

Immunoblotting

The liver samples were homogenized in RIPA buffer. 50 ug of proteins, determined using Biuret Reagent, were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After blocking, the membrane was incubated overnight with antibodies to anti-SMAD2/3 (Cell Signaling Technology, mAb #9523, 1:1000), p-SMAD2/3 (Cell Signaling Technology, mAb #9520, 1:1000), YAP/TAZ (Cell Signaling Technology, mAb #8418, 1:1000), anti-AMPK (Cell Signaling Technology, #2793, 1:1000), pAMPK (Cell Signaling Technology, mAb #2535, 1:1000), and ACTIN β as endogenous control (Abcam, ab8227, 1:1000) and then incubated with a secondary antibody. Band intensity was detected by chemiluminescence in GeneGnome equipment (Syngene) and evaluated by densitometry using Scion Image software (Scion Corporation).

Immunofluorescence

Cell proliferation was determined by ki67 immunofluorescence analysis. In brief, the liver frozen sections (7 µm) were fixed in 4% paraformaldehyde solution for 30 min, washed in PBS, and then, incubated in PBS solution containing 0.5% Triton X-100 and 0.05% SDS for 30 min. After washing, the slides were blocked with 1% albumin diluted in PBT (0.1M PBS +0.25% Triton X-100) for 2 h at room temperature. The slides were incubated with anti-KI67 (Abcam, ab15580, 1:100) primary antibody diluted in the blocking solution overnight at 4°C and secondary antibodies (Donkey anti-mouse FITC conjugated (1:250 dilution), Abcam, Cambridge, MA, United States). The fluorescence was visualized using the IMMU-Mount immunofluorescence medium (Thermo Fisher Scientific) with DAPI solution (Sigma, D9542, 1:2000) in a fluorescence optical microscope (LEICA DMI 4000B) and quantified with ImageJ software.

Cell Culture and Cycle Assay

Cell cycle analysis was performed using the AML12 mouse hepatocyte cell line (ATCC CRL-2254). The cells were maintained according to the work of Simino et al. (2021). Briefly, the cell were cultivated in the DMEM:HAM-F12 medium (1:1, 3.15 g/L glucose), with 10% FBS, 100 U/mL penicillin, 0.1 mg/ml streptomycin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml de selenium, and 40 ng/ml dexamethasone, and incubated at 37°C in 5% CO_2. The experiments were performed between passages 10 and 15. To determine the distribution of cell cycle stages, the cells were transfected with Let-7a mimic, inhibitor or scramble control sequence (10 nM, Ambion), or with miR-370 mimic, inhibitor, or scramble control sequence (10 nM, Ambion), along with Lipofectamine RNAimax (Invitrogen), in a serum-free culture

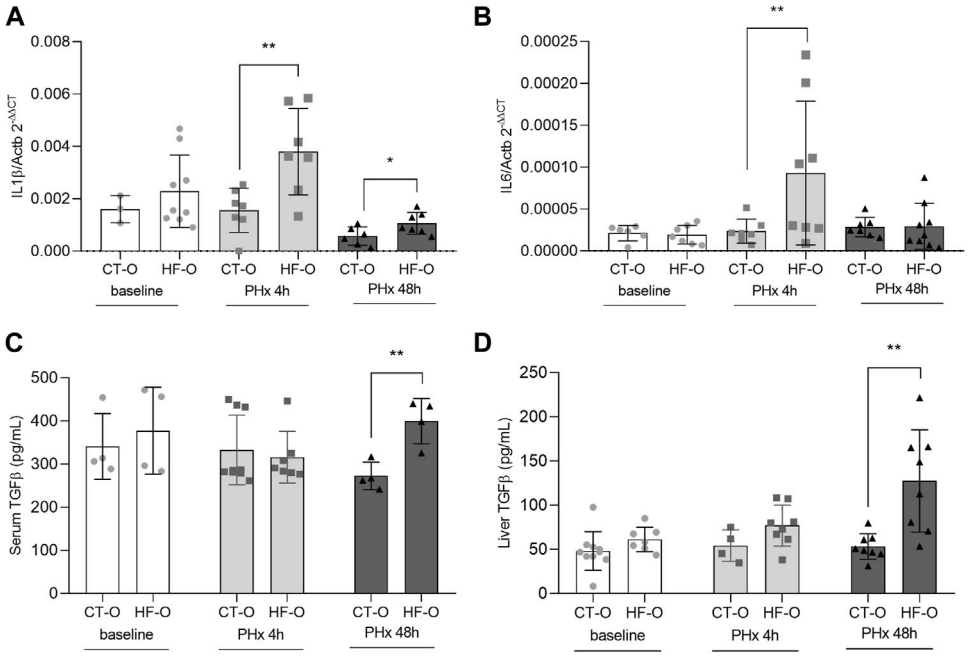

FIGURE 1 | Inflammatorymarkers of the offspring of control and obese dams after partial hepatectomy. Hepatic IL1β **(A)** and IL6 **(B)** gene expression (qPCR), serum TGFβ **(C)** and liver TGFβ **(D)** protein content (ELISA) from the offspring of control (CT-O) and obese (HF-O) dams, before (baseline) or after 4 (PHx 4 h) or 48 h (PHx 48 h) of partial hepatectomy surgery. Data are plotted as individual values (dot plots), means and SEM, represented by vertical bars. n = 3–9/group. $^{**}p \leq 0.005$ after Student's t-test (CT-O versus HF-O).

medium, for 24 h. The transfected hepatocytes were extracted and isolated according to the work of Severgnini et al. (2012). Cells (5×10^5 cells/mL) were resuspended in 20 μg/ml propidium iodide (ImmunoChemistry Technologies) diluted in 100 ul of PBS. Flow cytometric analysis was performed in two independent biological replicates from a sample pooling of two technical replicates, using a BD Accuri C6 cytometer on channels FL2 (585/40 nm) and FL3 (610/20 nm), with a total of 10,000 events counted in gate P2.

Statistical Analysis

The results are expressed as mean ± SEM. The Shapiro–Wilk test was used to confirm normality. Student's *t*-test was used to compare two groups, and Grubb's test was used to determine outlier. A significance level of $p \leq 0.05$ was established to assume differences between groups. The statistical analysis of each result is described in each figure legend. Data were analyzed using GraphPad Prism, version 8 (GraphPad Software Inc., United States).

RESULTS

Offspring Programmed by Maternal Obesity Have Slight Upregulation in Inflammatory Markers After Partial Hepatectomy

At mating, HF dams presented an obese phenotype, since they had higher body weight and adiposity, accompanied by elevated serum glucose, insulin, cholesterol (CHOL), and triacylglycerol (TAG) levels (**Supplementary Table S1**). Similarly, the offspring of obese dams (HF-O) had a dysmetabolic phenotype before the PHx procedure, as seen by higher body weight, and adiposity, as well as increased fasting glucose, serum insulin, and TAG levels compared to the offspring of control dams (CT-O) (**Supplementary Table S2**).

We further evaluated the inflammatory markers in the offspring after 4 and 48 h of PHx. HF-O had higher hepatic Il1b gene expression after 4 and 48 h PHx (**Figure 1A**) and upregulation in Il6 after 4 h PHx (**Figure 1B**). TGFβ was upregulated in both serum and the liver of HF-O 48 h following PHx (**Figures 1C,D**). Serum ALT and AST levels did not differ between groups for all time points studied, equally reflecting only the damage caused by the surgery (**Supplementary Table S3**).

Key Hepatic microRNAs and Their Predicted Targets Are Altered in the Liver of Offspring Programmed by Maternal Obesity After Partial Hepatectomy

We have previously reported that offspring programmed by maternal obesity during gestation and/or lactation have modulation in key hepatic microRNAs that leads to dysregulation in energy and lipid homeostasis, such as upregulation in miR-370 and Let-7a and downregulation of miR-122 (Benatti et al., 2014; Simino et al., 2017; Simino et al., 2021). In view of this, we performed an *in silico* analysis of predicted targets of miR-370, Let-7a, and miR-122 that might play an important role in liver regeneration (**Supplementary Table S4**).

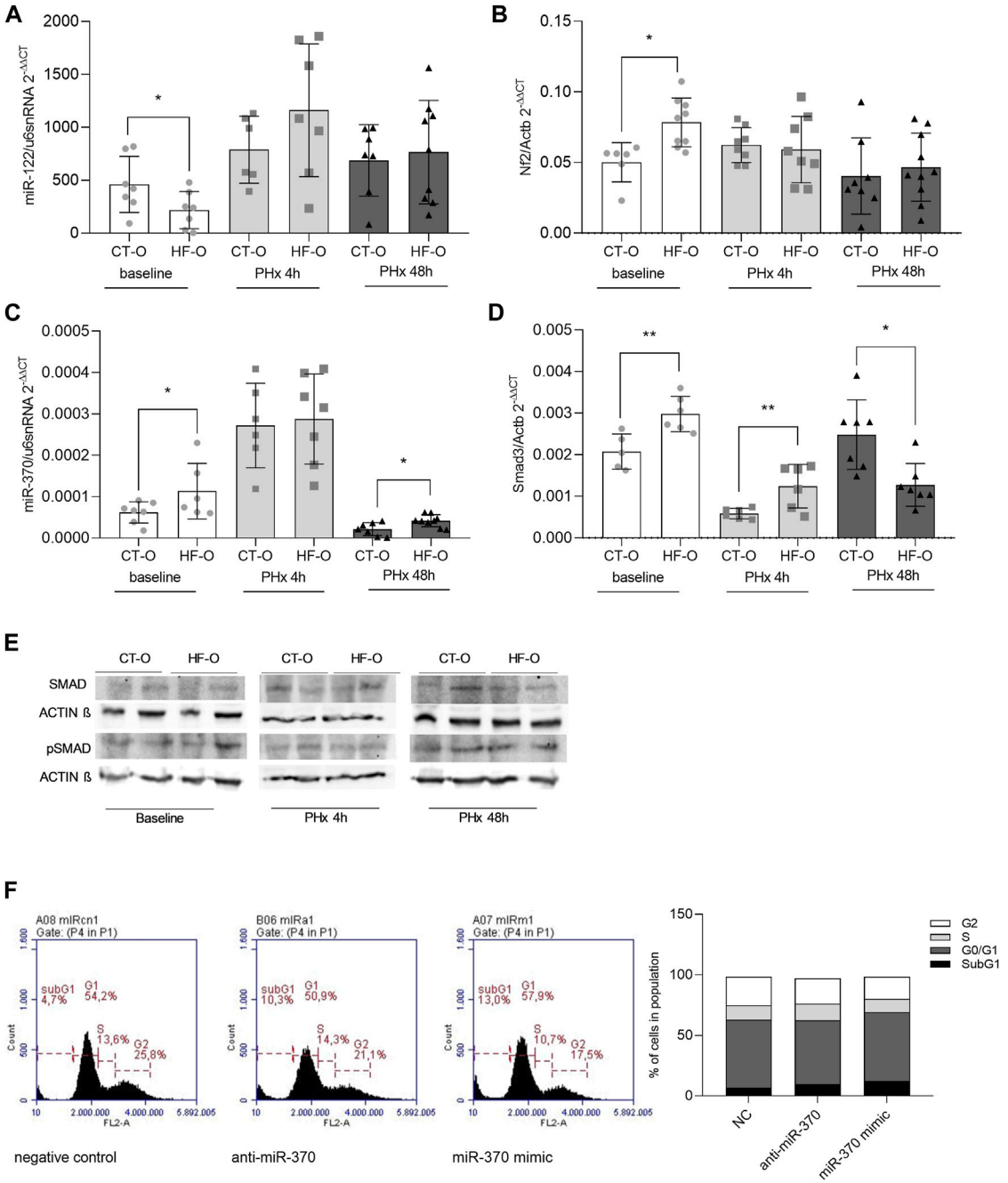

FIGURE 2 | Hepatic miR-122 and miR-370 pathways in the liver of the offspring of control and obese dams after partial hepatectomy. Hepatic miR-122-5p **(A)**, Nf2 **(B)**, miR-370-3p **(C)**, and Smad3 **(D)** gene expression (qPCR) and SMAD3 and pSMAD3 immunoblotting **(E)** from the offspring of control (CT-O) and obese (HF-O) dams, before (baseline) or 4 h (PHx 4 h) or 48 h (PHx 48 h) after partial hepatectomy surgery. Data are plotted as individual values (dot plots), means and SEM, represented by vertical bars. $n = 5$–10/group. $^{*}p \leq 0.05$ and $^{**}p \leq 0.005$ after Student's t-test (CT-O versus HF-O). Representative image and histogram of cell cycle distribution ($n = 2$) by flow cytometry in AML12 cells transfected with negative control, anti-miR-370, or miR-370 mimic **(F)**.

miR-122-5p is predicted to bind to *Nf2* mRNA 3′UTR (**Supplementary Table S3**). The *Nf2* gene codifies a protein that initiates a signaling pathway that leads to phosphorylation of the YAP/TAZ enzymatic complex that, in turn, exits the nucleus and prevents the transcription of proliferation-related genes (Nguyen-Lefebvre et al., 2021). As expected, baseline liver miR-122 levels were downregulated in HF-O offspring, whereas *Nf2* levels were upregulated. However, after 4 h or 48 h of PHx, miR-122 and *Nf2* levels in HF-O were comparable to CT-O (**Figures 2A,B**).

Mir-370-3p is predicted to pair with the targeting region of mRNAs 3′UTR from Smad3 and *Tgfβr2* (**Supplementary Table**

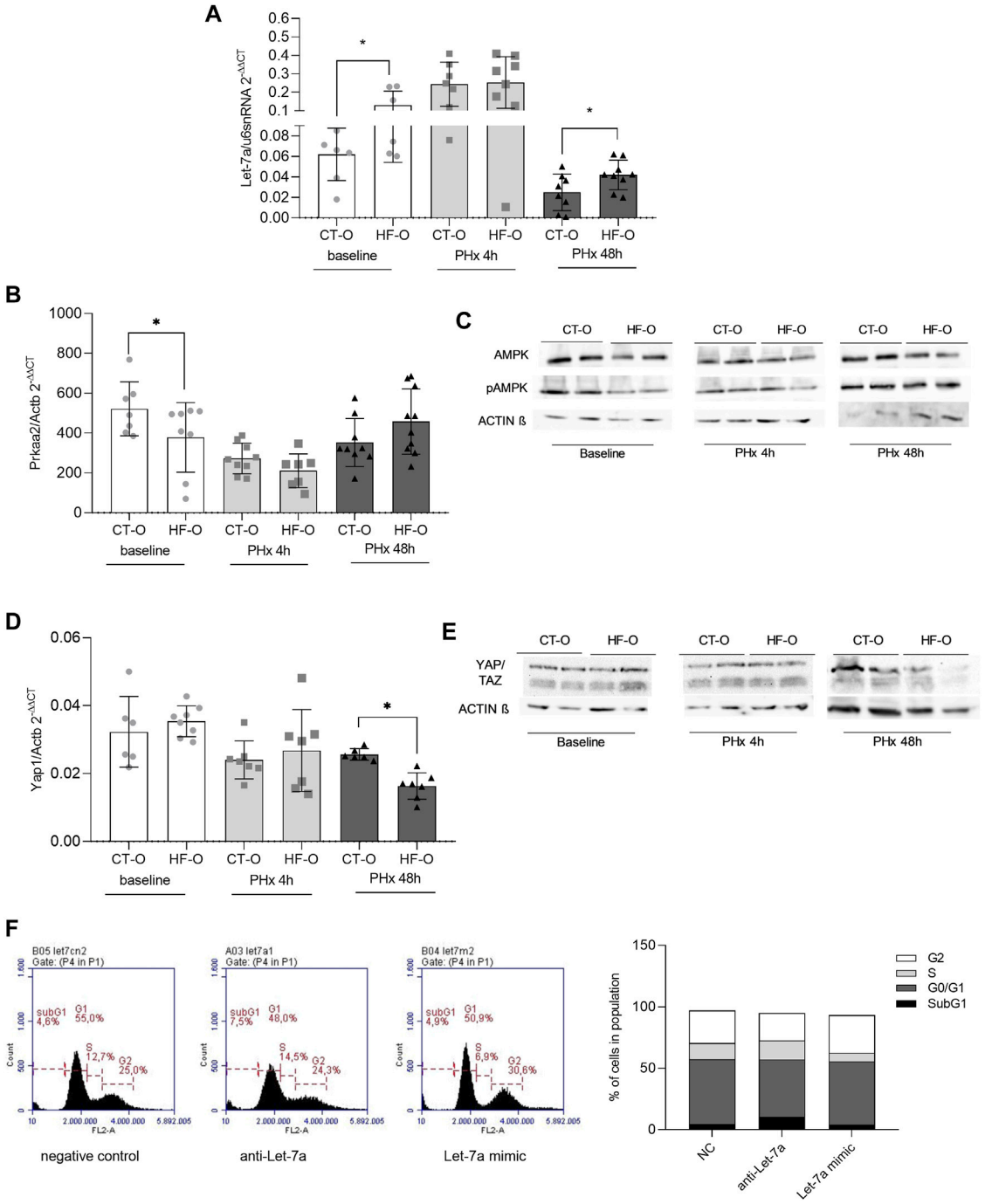

FIGURE 3 | Hepatic Let-7a pathways in the liver of the offspring of control and obese dams after partial hepatectomy. Hepatic Let-7a-5p **(A)** and Prkaa2 **(B)** gene expression (qPCR), AMPKa2 and pAMPKa2 immunoblotting **(C)**, YAP1 **(D)** gene expression (qPCR), and YAP/TAZ immunoblotting **(E)** from the offspring of control (CT-O) and obese (HF-O) dams, before (baseline) or 4 h (PHx 4 h) or 48 h (PHx 48 h) after partial hepatectomy surgery. Data are plotted as individual values (dot plots), means and SEM, represented by vertical bars. n = 5–10/group. *$p \leq 0.05$ and **$p \leq 0.005$ after Student's *t*-test (CT-O versus HF-O). Representative image and histogram of cell cycle distribution ($n = 2$) by flow cytometry in AML12 cells transfected with negative control, anti-Let-7a, or Let-7a mimic **(F)**.

S3). SMAD3 protein is downstream of the TGF-β receptor in the signaling pathway and acts by activating transcription factors of genes related to cellular proliferation (Nguyen-Lefebvre et al., 2021). Hepatic miR-370 was upregulated at baseline and 48 h after PHx in HF-O (**Figure 2C**). However, Smad3 mRNA showed a transient expression, since it was upregulated at baseline and 4 h after PHx, but downregulated 48 h after PHx in the liver of HF-O in comparison to CT-O (**Figure 2D**). While total-SMAD3 protein

levels tend to be downregulated in the liver of HF-O 48 h after PHx, the levels of pSMAD3 did not differ between HF-O and CT-O (**Figure 2E**). Moreover, the transfection of AML12 cells with anti-miR or mimic for miR-370 did not lead to significant alterations in cell cycle phases, although the presence of mimic for miR-370 reduced slightly the number of cells in the S-phase (11% miR-370 mimic *vs.* 12% NC) (**Figure 2F**).

We have previously shown that Let-7a regulates AMPKα2 levels (Simino et al., 2021). As expected, HF-O presented higher Let-7a and lower *Prkaa2* (the gene that encodes AMPKα2) hepatic transcript levels at baseline (**Figures 3A,B**). Also, AMPKα2 and pAMPKα2 protein levels were lower in the liver of HF-O compared to CT-O at baseline (**Figure 3C**). However, although Let-7a levels were upregulated in the liver of HF-O 48 h after PHX, AMPKα2 mRNA and protein levels did not differ between groups (**Figures 3A–C**). Let-7a is also predicted to bind to *Tgfβr1* and *Yap1* mRNA 3′UTR (**Supplementary Table S3**). YAP1 protein complexes with TAZ and acts as a transcription factor of genes related to cell proliferation (Nguyen-Lefebvre et al., 2021). YAP1 mRNA and protein levels did not differ between HF-O and CT-O at baseline. Nonetheless, 48 h after PHx, YAP1 gene expression and protein content were downregulated in the liver of HF-O (**Figures 3D,E**).

Flow cytometric analysis of AML12 cells showed that Let-7a levels may affect the hepatocytes' ability to progress in the cell cycle, since Let-7a anti-miR led to higher cell number at the S-phase, similar to a negative control (15.4% *vs.* 13.3%, respectively), while Let-7a mimic led to fewer cells in the S-phase compared to a negative control (6.9% Let-7a mimic *vs.* 13.3% NC) (**Figure 3F**).

Hepatocyte Proliferation and Liver Mass Recovery of Offspring Programmed by Maternal Obesity After Partial Hepatectomy

To determine the liver regeneration capacity from the offspring of control and obese dams, we performed ki67 labeling of liver sections 48 h after PHx. HF-O had lower ki67 labeling 48 h after PHx, compared to CT-O (**Figure 4A**). However, the liver mass was similar between CT-O and HF-O 48 h after PHx because the liver/body weight ratio did not differ between the groups (**Figure 4B**).

DISCUSSION

This study sought to explore the relationship between miR-122, miR-370, and Let-7a expression with liver regeneration capacity in metabolically programmed offspring. We have confirmed that the development of an obese phenotype by HFD-fed dams leads the offspring to dysregulation of hepatic miR-122, miR-370, and Let-7a expression patterns and the predicted target genes involved with proliferation and regeneration. We have also shown altered dynamics of the cell cycle in hepatocytes transfected with mimic for Let-7a and miR-370, which reinforces the role of these miRNAs in the regenerative process. Although both the microRNAs and their targets were altered into the offspring of obese dams, most of them were comparable to CT-O after liver resection through ⅔ partial hepatectomy. In addition, despite the Ki67 labeling reflecting some delay in cell proliferation in HF-O, the liver mass recovery was not compromised, supporting the hypothesis of epigenetic reprogramming mechanism. To our knowledge, this is the first study that demonstrates the impact of obesogenic metabolic programming on epigenetic regulators that leads to the dysregulation in energy and lipid homeostasis and its role in liver regeneration.

There is much evidence that maternal obesity and/or high-caloric diet consumption during pregnancy and lactation can promote adverse metabolic outcomes in offspring (Zambrano et al., 2010; Alfaradhi et al., 2014; Benatti et al., 2014; Melo et al., 2014; Gaillard, 2015; Fante et al., 2016; Simino et al., 2017; Costa et al., 2020). Among the most well-characterized alterations in the liver of the offspring of obese dams, the development of NAFLD and its interconnection with insulin resistance has been widely explored (Watt et al., 2019; Tanase et al., 2020).

The liver is the main metabolic site and an important organ in immunity, and challenges in the perinatal period can lead to functional adaptations (Nakagaki et al., 2018). Maternal overnutrition during development can disturb the offspring's hepatic function. In an elegant study developed by McCurdy et al. (2009), it was shown in non-human primates that maternal HFD consumption induced steatosis, oxidative stress, and apoptosis in liver fetuses, compromising liver function (McCurdy et al., 2009). The same was observed by Heerwagen et al. (2013) in mice at embryonic day 18.5 (Heerwagen et al., 2013). Additionally, premature weaning *per se* profoundly impacted the expression of several hepatic metabolic pathways leading to reprogramming in liver structure and function (Nakagaki et al., 2018).

The liver has a unique ability to regenerate (Mao et al., 2014), though the excessive presence of ectopic fat is a limiting factor for liver regeneration (Truant et al., 2013; Amini et al., 2016). Notably, our recent study showed that the offspring of obese dams have delayed regenerative process after partial hepatectomy; however, they seem to be programmed by maternal overnutrition to overlap the metabolic damage and promote cellular proliferation to ensure survival (Fante et al., 2021). However, this previous study did not assess the epigenetic modulation in the offspring of obese dams and the role of miRNAs in liver regeneration. The current study demonstrates that partial hepatectomy can induce changes in miRNA that impact lipid metabolism in liver offspring, reversing the expression of target genes involved in the regenerative process.

We have previously shown that maternal obesity induced by HFD consumption during gestation and/or lactation drives miRNA modulation in the liver of the offspring, which predisposes them to ectopic lipid accumulation and systemic metabolic alterations, consistent with NAFLD (Benatti et al., 2014; Simino et al., 2017; Simino et al., 2021). The offspring of obese dams showed upregulation of hepatic miR-370 and downregulation of miR-122 (Benatti et al., 2014). miR-122 is the most abundant miRNA in the liver that targets key genes involved in lipid and insulin homeostasis, thus playing an

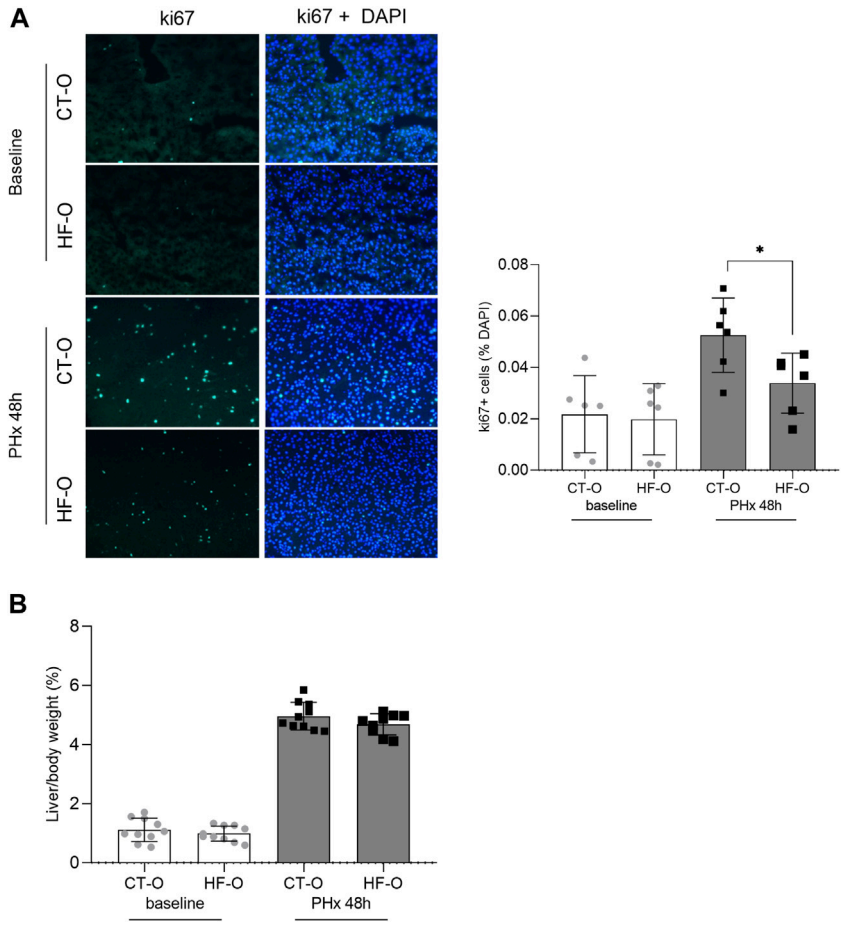

FIGURE 4 | Liver regeneration capacity from the offspring of control and obese dams 48 h after partial hepatectomy. Immunofluorescence of hepatic ki67 content **(A)** and liver/body weight ratio **(B)** from the offspring of control (CT-O) and obese (HF-O) dams, 48 h (PHx 48 h) after partial hepatectomy surgery. Data are plotted as individual values (dot plots), means and SEM, represented by vertical bars. n = 6–10/group. $^{*}p \leq 0.05$ and $^{**}p \leq 0.005$ after Student's t-test (CT-O versus HF-O).

important role in general metabolic functions (Hsu et al., 2012; Tsai et al., 2012; Yang et al., 2012). miR-370 is also of great importance for liver metabolic homeostasis since it targets genes involved in fatty acids oxidation, such as *Cpt1a* and *Acadvl*, and controls miR-122 levels (Iliopoulos et al., 2010; Benatti et al., 2014; Simino et al., 2017). Similar results were obtained in a prenatal dexamethasone exposure (PDE) model. Liu et al. (2021) found increased lipid accumulation and molecular clues of NAFLD in the liver of adult male rat offspring consistent with low hepatic miR-122 expression.

Using bioinformatics approaches, we identified that *Nf2* (Neurofibromin 2) is a predicted target of miR-122. NF2 is known as a key component in the HIPPO signaling cascade, the major regulator of organ size/growth (Lee et al., 2021; Nguyen-Lefebvre et al., 2021). Upon acute tissue injuries, such as liver resection, NF2 no longer mediates YAP/TAZ (Yes-associated protein and transcriptional coactivator with PDZ-binding motif) complex phosphorylation and allows its recruitment to the nucleus, where it functions as a transcription factor for repair and regeneration processes (Lee et al., 2021; Nguyen-Lefebvre et al., 2021). At baseline conditions, HF-O had lower hepatic miR-122 levels, while *Nf2* gene expression was upregulated, which is an additional indicator that miR-122 can act as an *Nf2* repressor. It was already shown that transcripts associated with the Hippo signaling pathway, such as NF2, may be upregulated in obese mice and their descendants (An et al., 2017). However, here, after PHx, neither miR-122 nor *Nf2* levels were different in the liver of HF-O in comparison to CT-O. Although Nf2 gene expression dysregulation could be involved in NAFLD pathogenesis and progression to HCC (Ardestani et al., 2018), the reduction in the transcript levels in the HF-O group 48h after PHx, compared to baseline, may be an indication that the pathway that controls cell repair is more permissive in this group after mechanical injury, similar to the CT-O. This result is supported by the findings of cyclin D1 content in the same model in our previous work (Fante et al., 2021). Xiao et al. (2005) suggested that the product of the Nf2 gene inhibits cell cycle progression through Cyclin D1 suppression since Nf2 gene silencing results in upregulation of cyclin D1 and S-phase entry. We have shown that despite cyclin D1 showing a tendency to decrease in the HF-O group 48hs after PHx, the protein content still increases greatly in comparison to

the baseline, being similar to the CT-O group and allowing the cell to enter the S-phase of the cycle, even with some delay (Fante et al., 2021).

The *Yap1* gene, of the YAP/TAZ complex, is reported here as a predicted target of Let-7a. We have previously shown that Let-7a is upregulated in the liver of the offspring of obese dams, and it targets *Prkaa2*, the gene that encodes AMPKα2 protein, leading to the disruption of the metabolic homeostasis (Simino et al., 2021). The Let-7 family is mainly known for its ability to regulate the expression of proteins involved in cell development, such as LIN28 (Reinhart et al., 2000) which has an important role in tissue repair (Shyh-Chang et al., 2013). The Let-7/Lin28 axis has been shown to be vital for the maintenance of the cell cycle in several conditions, and Let-7 overexpression may be detrimental for leading to the loss of cell growth and apoptosis induction (McDaniel et al., 2016). Let-7a levels were upregulated in the liver of HF-O at baseline and 48 h after PHx. However, attenuation on *Let-7* 4 h after PHx could confer the ability of the hepatic tissue to repair in circumstances of mechanical insult.

Lee et al. (2021) reported that several microRNAs may be able to suppress *Yap1* (miR-497, miR-186, miR-590-5p, miR-424-5p, miR-506, miR-132, mir_520c-3p, miR-375, miR-125b, miR-9-3p, miR-223, and miR-338-3p). On the other hand, Chaulk et al. (2014) suggested that YAP/TAZ could act as a transcription factor for Let-7 biogenesis since in MCF10A cells, Let-7 levels accumulated upon the loss of the nuclear YAP/TAZ. However, according to our research, this is the first study to suggest that Let-7a may pair with *Yap1*. *Yap1* gene expression and YAP/TAZ protein content did not differ between CT-O and HF-O at baseline, but 48 h after PHx, both *Yap1* expression and YAP/TAZ content were lower in the liver of HF-O, which would be consistent to *Let-7a* increasing.

The YAP/TAZ complex also interacts with other proteins and pathways involved in cellular proliferation and tissue regeneration. The dephosphorylation of YAP/TAZ and its consequent translocation to the nucleus allow it to form a complex with SMADS, which are key components of the TGFβ signaling pathway (Qin et al., 2018). In the current study, while *Tgfbr1* is shown to be a predicted target of Let-7a, *Tgfbr2* and *Smad3* are predicted targets of miR-370. TGFβ is known as an antiproliferative factor that plays an important role in the termination phase of the liver regeneration following partial hepatectomy since when the tissue has already been restored to its original size, hepatocyte growth must be inhibited to maintain constant liver mass and function (Qin et al., 2018; Nguyen-Lefebvre et al., 2021). On the other hand, high levels of TGFβ are associated with increased BMI (body mass index), metabolic damage, and higher serum glucose and insulin levels (Yadav et al., 2011; Tan et al., 2012). Although baseline TGFβ levels in HF-O were similar to the CT-O, 48 h after PHx, HF-O had higher TGFβ in both serum and the liver. Also, while the liver mass did not differ between groups, ki67 staining was slightly lower in the liver of HF-O 48 h after PHx, so we can correlate the upregulation of serum and liver TGFβ with a presumable marker of an efficient termination phase of the liver regeneration process.

In vitro experiments of gain and loss of function with antagomiR and mimic for Let-7a and miR-370 showed that these miRNAs are involved in the cell proliferation process since they were able to modulate different stages of the cell cycle. The transfection of hepatocytes with mimic for both Let-7a and miR-370 led to a reduction in the percentage of cells in the S-phase of the cell cycle. However, it is important to emphasize that even with some delay, the triggering of the regenerative process in the liver of offspring of obese dams still occurs as recently described by our laboratory (Fante et al., 2021).

In conclusion, repair mechanisms in metabolic programmed offspring occur through epigenetic reprogramming, thus ensuring tissue regeneration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee for Ethics in Animal Experimentation at the State University of Campinas-UNICAMP (protocol #4594-1/2017).

AUTHOR CONTRIBUTIONS

LS, MF, TF, and CP: data acquisition, formal analysis, methodology. LS, CP, MT, LI-S, MM, and AT: manuscript writing, reviewing, and editing. AT: conceptualization, data curation, project administration, and resource management. All authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.830009/full#supplementary-material

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Maternal Protein Restriction Alters the Expression of Proteins Related to the Structure and Functioning of the Rat Offspring Epididymis in an Age-Dependent Manner

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Nutrition is an environmental factor able to activate physiological interactions between fetus and mother. Maternal protein restriction is able to alter sperm parameters associated with epididymal functions. Since correct development and functioning of the epididymides are fundamental for mammalian reproductive success, this study investigated the effects of maternal protein restriction on epididymal morphology and morphometry in rat offspring as well as on the expression of Src, Cldn-1, AR, ER, aromatase p450, and 5α-reductase in different stages of postnatal epididymal development. For this purpose, pregnant females were allocated to normal-protein (NP—17% protein) and low-protein (LP—6% protein) groups that received specific diets during gestation and lactation. After weaning, male offspring was provided only normal-protein diet until the ages of 21, 44, and 120 days, when they were euthanized and their epididymides collected. Maternal protein restriction decreased genital organs weight as well as crown-rump length and anogenital distance at all ages. Although the low-protein diet did not change the integrity of the epididymal epithelium, we observed decreases in tubular diameter, epithelial height and luminal diameter of the epididymal duct in 21-day-old LP animals. The maternal low-protein diet changed AR, ERα, ERβ, Src 416, and Src 527 expression in offspring epididymides in an age-dependent manner. Finally, maternal protein restriction increased Cldn-1 expression throughout the epididymides at all analyzed ages. Although some of these changes did not remain until adulthood, the insufficient supply of proteins in early life altered the structure and functioning of the epididymis in important periods of postnatal development.

Keywords: maternal protein restriction, epididymis, hormone receptors, src, CLDN-1, postnatal development

1 INTRODUCTION

One of the first concepts to emerge in the literature relating the conditions of the intrauterine environment to changes in progeny development was the "fetal origin of diseases" theory (Barker, 1995a). The "fetal origin" hypothesis proposes that changes in fetal nutrition and hormonal status result in adaptations during development capable of altering the structure, physiology and metabolism of the embryo, thus predisposing it to cardiovascular, metabolic and endocrine diseases in adult life (Barker, 1995b; 2007). Currently, the concept that intrauterine environmental conditions are able to influence the establishment of adulthood diseases is known as the Developmental Origins of Health and Disease (DOHaD) (Jazwiec and Sloboda, 2019).

It is well established that maternal nutrition has a significant impact on development and fetal health, since during gestation and lactation, the fetus depends exclusively on the mother to supply its nutritional requirements (McArdle et al., 2006; Jazwiec and Sloboda, 2019; Lindsay et al., 2019). Therefore, nutrition during pregnancy is an environmental factor able to activate physiological interactions between the fetus and mother that are often mediated through hormonal signaling and may cause epigenetic alterations in genes that regulate the target tissues of these hormones. These interactions may modify fetal growth and metabolic character, establishing the basis for several diseases when there is inconsistency between gestational and postnatal nutrient availability (Fleming et al., 2015).

Hormonal signaling changes during sensitive periods of development may alter the development of specific fetal tissues, lead to long-lasting changes in tissue sensitivity to hormones or even change hormone secretion (Godfrey and Barker, 2000; Console et al., 2001; Peixoto-Silva et al., 2011; Rinaldi et al., 2018). Rats subjected to maternal protein restriction during intrauterine development show changes in testosterone, estradiol and aldosterone concentration that result in significant impacts on organs and functions of the genital system (Zambrano et al., 2005; Colombelli et al., 2017; Santos et al., 2018; Cavariani et al., 2019).

The epididymis is an androgen-dependent organ anatomically divided into the initial segment, caput, corpus, and cauda. Each epididymal region is responsible for characteristic functions such as secretion, endocytosis, absorption and acidification, which lead to the establishment of a specific intraluminal environment suitable for the concentration and maturation of the spermatozoa produced by the testes (Cosentino and Cockett, 1986; Hermo and Robaire, 2002; Gatti et al., 2004; Robaire and Hinton, 2015).

The epididymis originates from the proximal part of the Wolffian duct, acquiring a coiled appearance by the influence of testosterone (Moore et al., 2016). The enzyme 5α-reductase mediates the conversion of testosterone to dihydrotestosterone (DHT), which is the most active regulator of epididymal cell function being five to ten times more potent than testosterone with a higher affinity to androgen receptor (AR) (Gloyna and Wilson, 1969; Cohen et al., 1981; Rhoden et al., 2002; Hackel et al., 2005; Traish et al., 2015). Both testosterone and DHT bind to the AR being responsible for activating different processes (Silverthorn, 2016).

Estrogen is also actively involved in the development and functions of the epididymis (Hess and Zhou, 2002; Cooke et al., 2017; Hess et al., 2021). In immature males, the main source of estrogen is the Sertoli cells, while in adults, germ cells show high expression of the enzyme aromatase cytochrome p450, which converts androgen into estrogen (Nitta et al., 1993; Hess et al., 1995; Janulis et al., 1998; Ghosh et al., 2009; Russell and Grossmann, 2019). Estrogens mediate their effects by binding to the nuclear receptors termed ERα and ERβ in the epididymis (Kuiper et al., 1996; Pelletier et al., 2000; Zaya et al., 2012).

A maternal low-protein diet during gestation and lactation is able to induce testicular, prostatic and sperm changes in adult animals (Zambrano et al., 2005; Toledo et al., 2011; Rodriguez-Gonzalez et al., 2012; Rodriguez-Gonzalez et al., 2014; Colombelli et al., 2017; Santos et al., 2018). Regarding sperm alterations, it has been observed that maternal protein restriction caused alterations mainly associated with epididymal functions such as sperm motility, viability and concentration (Toledo et al., 2011; Rodriguez-Gonzalez et al., 2014). However, although studies have shown effects of protein restriction associated with epididymal functions, the causes of these alterations have not yet been entirely clarified.

During gestation, an increase in protein turnover occurs to enable rapid embryo growth; therefore, adequate intake of protein at this stage is recommended (Jolly et al., 2004). Although some effects of protein restriction are mediated by hormonal effects, several others are direct consequences of changes in substrate availability.

Srcs are nonreceptor protein kinases that act in multiple cellular environments, playing key roles in the regulation of signal transduction through several cell surface receptors (Parsons and Parsons, 2004). In the epididymis, Srcs stand out as regulators of epididymal development in addition to playing important roles in spermatozoa changes that occur during epididymal transit and sperm maturation within the organ (Krapf et al., 2012; Xu et al., 2016).

The structure and integrity of the blood–epididymal barrier are essential for the maintenance of epididymal intraluminal environment specificity (Hoffer and Hinton, 1984; Dufresne and Cyr, 2007). In this context, the claudins (Cldns) are a family of transmembrane proteins that are essential components of the tight junctions that compose this barrier (Gregory and Cyr, 2006; Cyr et al., 2007). Although several claudins integrate tight junctions, studies have demonstrated the presence of Cldn-1 in all regions of the rat epididymis. In addition, Cldn-1 appears throughout all interfaces of adjacent epithelial cells and throughout all basal plasma membrane extensions, suggesting that Cldn-1 plays a role as an adhesion molecule (Gregory et al., 2001; Dufresne and Cyr, 2007; Kim and Breton, 2016). Cldn-1 knockout mice die of dehydration soon after birth due to lack of the epidermal barrier, thus demonstrating that Cldn-1 is indispensable for survival and cannot be replaced by any of the other tight junction proteins (Furuse et al., 2002).

TABLE 1 | Composition of diets offered to animals during gestation and lactation.

Components *	Normoprotein diet (17%)	Low-protein diet (6%)
Casein (84% of protein)**	202.00	71.50
Cornstarch	397.00	480.00
Dextrin	130.50	159.00
Sucrose	100.00	121.00
Soy oil	70.00	70.00
Fiber (microcellulose)	50.00	50.00
Mineral Blend ***	35.00	35.00
Vitamin Blend ***	10.00	10.00
L-cystine	3.00	1.00
Choline chloride	2.50	2.50

** Diet for the gestation phase in rodents - AIN-93G.*
*** Corrected values according to protein content in casein.*
**** According to AIN-93G.*

Insufficient protein intake in a large proportion of the human population due to cultural or economic reasons is a global concern. For this reason, the model of protein restriction remains one of the most characterized early growth restriction models studied until now (Fleming et al., 2015; Semba, 2016; Herring et al., 2018). Maternal protein restriction is able to alter sperm parameters associated with epididymal function, such as sperm motility, viability and concentration. Since the correct development and functioning of the epididymis are fundamental for mammalian reproductive success, this study investigated the effects of maternal protein restriction on epididymal morphology and morphometry in rat offspring as well as on the expression of Src, Cldn-1, AR, ERα, ERβ, aromatase p450, and 5α-reductase in different stages of postnatal epididymal development.

Considering the direct effects of maternal protein restriction related to changes in substrate availability and hormonal signaling, we hypothesized that the sperm alterations associated with the correct functioning of epididymis previously observed in this experimental model are related to differential expression of membrane proteins and hormone receptors essential for the epididymal performance in specific periods of postnatal development.

2 MATERIALS AND METHODS

2.1 Animals and Experimental Design

Wistar rats 45 days in age (male $n = 20$; female $n = 38$) were obtained from the Central Biotherium, Institute of Biosciences/Campus of Botucatu, UNESP–São Paulo State University. The animals were housed in polyethylene cages (43 × 30 × 15 cm) lined with an autoclaved pine sawdust substrate under controlled conditions of temperature and light (12-h light/dark cycle). The animals were maintained with free access to water and commercial solid food for rodents.

For mating, two sexually receptive females and one breeder male rat at 95 days of age were kept in maternity boxes overnight. The next morning, pregnancy was confirmed by the presence of spermatozoa in vaginal smears; this day was designated gestational day (GD) 0. Pregnant females were housed individually in standard rat cages and divided into a low-protein (LP) group ($n = 19$) and a normal-protein (NP) group ($n = 19$). The LP mothers were fed a low-protein diet (6% protein), while the NP mothers were fed a normal-protein diet (17% protein), both provided from Pragsoluções Biociências (Jaú, SP, Brazil). The NP and LP diets were prepared following the recommendation by the American Institute of Nutrition (AIN 93-G) (Reeves et al., 1993). The diets are isocaloric and widely used for the study of maternal protein restriction (Rinaldi et al., 2013; Colombelli et al., 2017; Santos et al., 2018; Cavariani et al., 2019). Both groups received their respective diets ad libitum (**Table 1**).

The normal-protein and low-protein diets were offered to the indicated groups during gestation and lactation, from GD 0 until the offspring were weaned at postnatal day 21. To ensure equal availability of nourishment, only eight pups per litter (preferably males) were maintained with each mother. After weaning, the LP and NP male offspring received the standard diet for rodents until the age of 21 (NP, $n = 17$; LP, $n = 22$), 44 (NP, $n = 12$; LP, $n = 10$) and 120 (NP, $n = 13$; LP, $n = 12$) days, when they were decapitated and their blood and epididymides collected (**Figure 1**). Each individual was chosen from different litters to ensure a representative sampling. The ages of the animals at euthanasia were chosen based on three different phases of the epididymis postnatal development: at 21 days of age, peak epididymal cell differentiation occurs; at 44 days of age, the final period of epididymal differentiation and the beginning of epididymal expansion occurs; and at 120 days of age, the epididymides are well differentiated, and the animals are considered sexually mature (Picut et al., 2018).

The experimental procedures were approved by the Ethics Committee on Animal Experimentation (EAEC) of the Institute of Biosciences of Botucatu (number 797-CEUA) in addition to being in accordance with the Ethical Principles on Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2 Gestational Performance

Gestational performance is an important parameter in studies that use maternal malnutrition as an experimental model. This parameter is assessed on the day of offspring birth (PND 1) and presents relevant information regarding gestation and offspring development.

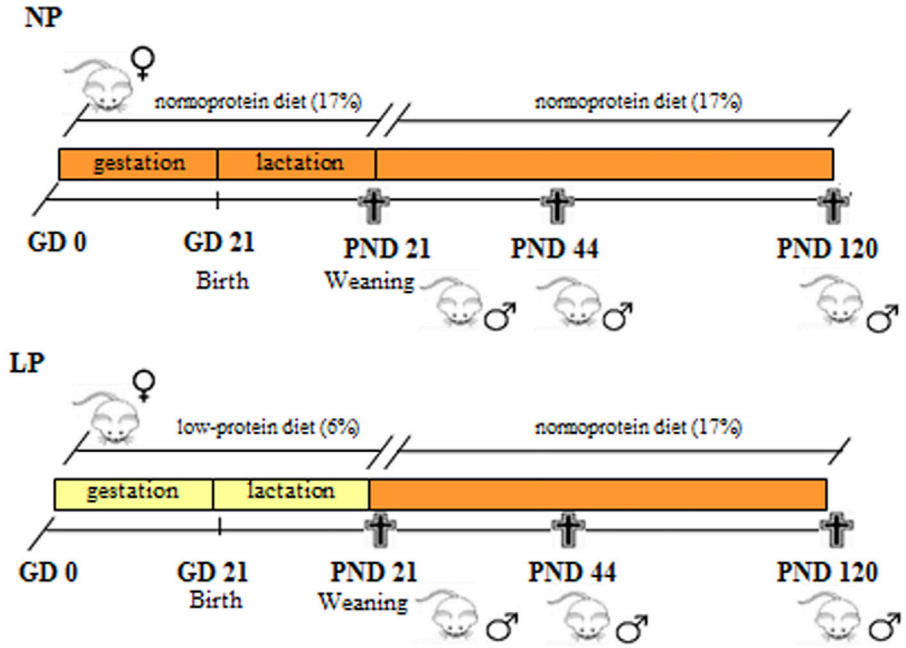

FIGURE 1 | Experimental design. Pregnant rats received a low-protein diet (LP group) or a normal-protein diet (NP group) ad libitum from the GD 0 until PND 21 (during gestation and lactation). After weaning, male pups from both groups received a standard diet until PNDs 21, 44 and 120.

In PND 1, male pups from each litter were separated, weighed individually and had the anogenital distance (AGD) and crown-rump length (CRL) recorded using a digital caliper (Western®). Relative AGD was obtained by dividing the absolute AGD by the CRL. For each litter of NP and LP groups, the number of male and female pups were counted in addition to the total number of pups (data not shown).

2.3 Hormonal Assay

Through narcosis induced in a CO_2 chamber, the animals were anesthetized and then decapitated (for cervical vessel rupture), and the blood was collected. Blood serum was obtained by centrifugation at 14,000 rpm for 20 min at 4°C. Serum samples were assayed for estradiol levels using chemiluminescence with a specific kit provided by Beckman Coulter, Inc. (REF B84493, Brea, CA, United States). The lower and higher limits of detection for estradiol were 0.017 ng/dl and 6.9 ng/dl, respectively. All samples were assayed at the same time to prevent interassay variation.

2.4 Morphological and Morphometrical Analyses of the Epididymis

Following euthanasia, the right epididymides were individually collected, immediately fixed in 10% buffered formalin (0.1 M phosphate buffer, pH 7.3) for 24 h and then washed in running water for 24 h. Then, the epididymides were dehydrated in a graded series of ethanol solutions, diaphanized in N-butyl alcohol and embedded in Paraplast (Paraplast Plus, St. Louis, MO, United States). Epididymal **sections 5** μm thick were made using a LEICA RM 2165 μm (Leica Biosystems, Nußloch, Germany). Four blocks were cut for the NP and LP groups at each age; the blocks from the animals at PND 21 were cut into serial sections, while the blocks from the animals at PNDs 44 and 120 were cut into semiserial sections.

For all ages (21, 44 and 120 days), four slides from each of the four animals of each group (LP and NP) were stained with hematoxylin and eosin (H&E). The slides were scanned with a 3DHistech Pannoramic MIDI, analyzed for epithelial and interstitial integrity and then photographed using the Pannoramic Viewer program.

Morphometric analyses were conducted by adapting the procedures described by Serre and Robaire (1998). For this technique, the same slides used for analysis of epididymal morphology were analyzed. Briefly, the epithelial height, luminal diameter and tubular epididymal diameter was measured in at least 10 transverse sections of epididymal tubules in each of the epididymal regions using the Pannoramic Viewer program.

2.5 Immunohistochemistry

For immunohistochemistry, sections of epididymides from LP and NP animals (n = 4 animals/group at each age) were deparaffinized (40 min in the oven at 60 °C), incubated in xylene and hydrated with decreasing concentrations of ethanol. Antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) in a water bath for AR and ERα at PNDs 21 and 44 and in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker for AR and ERα at PND 120 and for ERβ at all ages. After endogenous peroxidase was blocked (H2O2, 0.3% in

methanol), the tissues were incubated with 3% BSA for 1 h. The sections of epididymis were incubated overnight at 4 °C with 1: 100 dilutions of the following primary antibodies in 1% BSA: anti-AR (Millipore, Temecula, CA, United States), anti-ERα (Millipore, Temecula, CA, United States) and anti-ERβ (Millipore, Temecula, CA, United States). Early the next morning, the sections were washed with PBS buffer and then incubated with 1:200 dilutions of a peroxidase-conjugated anti-Rb secondary antibody (Sigma, St. Louis, MO, United States) for AR and ERα at PNDs 21 and 44 and a biotinylated anti-Rb secondary antibody (Sigma, St. Louis, MO, United States) for AR and ERα at PND 120 and for ERβ at all ages in 1% BSA for 2 h. The sections labeled with biotinylated antibodies were incubated for 45 min with an ABC complex (ABC Vectastain® kit, Burlingame, CA, United States) and subsequently washed with PBS. The immunoreactive components were reacted with diaminobenzidine (DAB; Sigma, St. Louis, MO, United States), and counterstaining was performed with hematoxylin. Finally, a 3D Histech Pannoramic MIDI was used to scan the slides, which were photographed and analyzed using the Pannoramic Viewer program. Negative controls were obtained from each reaction using 1% BSA and omitting the primary antibody in the overnight incubation step.

2.6 Western Blot Analysis

The left epididymides from NP and LP animals at the ages of 21, 44, and 120 days ($n = 5$) were divided into the initial segment plus caput (SI + CP) and the corpus plus cauda (CO + CD). The samples were homogenized with RIPA lysis buffer (Bio-Rad, Hercules, CA, United States) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO, United States). Subsequently, the homogenate was centrifuged at 14,000 rpm for 20 min to remove the cell debris, and the supernatant was then collected. Total protein concentrations were measured using the Bradford colorimetric method (Bradford, 1976). Afterwards, 70 μg of protein was added to 1.5X Laemmli buffer, and the individual proteins were then separated by 4–15% polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 120 V. Following electrophoresis, the proteins were electrotransferred to nitrocellulose membranes in a wet system at 350 mA. The membranes were blocked with TBS-T solution containing 3% milk (Molico®) for 1 h at room temperature. The membranes were incubated overnight at 4°C with the following primary antibodies diluted in TBS-T: anti-AR (1:1,000 dilution; Millipore, Temecula, CA, United States), anti-ERα (1:200 dilution; Millipore, Temecula, CA, United States), anti-ERβ (1:300 dilution; Millipore, Temecula, CA, United States), anti-Src 416 (1:500 dilution; Cell Signaling, Danvers, MA, United States), anti-Src 527 (1:1,000 dilution; Cell Signaling, Danvers, MA, United States), anti-Cldn-1 (1:1,000 dilution; Thermo Fisher Scientific, Rockford, IL, United States) and anti-β-actin (1:800 dilution; Santa Cruz, Santa Cruz, CA, United States). Early the next morning, the membranes were washed with TBS-T solution and then incubated with a 1:2000 dilution of an anti-Rb secondary antibody for AR, ERα, ERβ and Cldn-1 (Sigma, St. Louis, MO, United States), a 1:5,000 dilution of an anti-Rb secondary antibody for Src 416 and Src 527 (Sigma, St. Louis, MO, United States) and an anti-goat secondary antibody (1:6,000 dilution; Sigma, St. Louis, MO, United States) diluted in TBS-T for 2 h before being washed with TBS-T solution. Subsequently, the immunoreactive bands were developed using a chemiluminescence kit (Amersham ECL™ Western Blotting Detection Reagent Select) from GE Healthcare® and analyzed semiquantitatively by optical densitometry with ImageJ analysis software for Windows. The values obtained for each band of AR, ERα, ERβ, Src 416, Src 527, and Cldn-1 were normalized to the β-actin density, and the data are presented as the mean ± S.E.M. The immunoblotting data are presented as optical densitometry index (% band intensity).

TABLE 2 | Body weight, crown-rump length and absolute and relative anogenital distance of males at birth (PND 1). NP, $n = 17$ litters/group. LP, $n = 19$ l/group. Values expressed as means ± S.E.M. *$p < 0.05$. Student t-teste was used to asses significance of differences in parametric data (a) and Mann-whitney was used to asses significance of differences in non-parametric data (b).

Parameters	*NP*	*LP*
Number of male pups	5.29 ± 0.43	5.47 ± 0.52[a]
Body weight (g)	6.33 ± 0.12	5.79 ± 0.10[a*]
Anogenital distance (mm)	2.99 ± 0.03	2.59 ± 0.05[b*]
Crown-rump length (mm)	49.13 ± 0.32	47.46 ± 0.21[a*]
Relative anogenital distance (mm)	0.061 ± 0.001	0.055 ± 0.001[b*]

2.7 Statistical Analysis

GraphPad Prism® software (version 5.00, Graph Pad, Inc., San Diego, CA) was used to perform the statistical analyses. At all analyzed ages, comparisons between the LP and NP groups were performed using the Mann-Whitney test for nonparametric data and Student's t-test for parametric data. All data are presented as the mean ± S.E.M., and statistical significance was set at $p < 0.05$.

3 RESULTS

3.1 Maternal Low-Protein Diet Promotes Changes in Gestational Performance as Well as Genital Organ Weight in Male Offspring

Maternal protein restriction during gestation and lactation did not alter the number of male pups but significantly decreased the body weight of the pups at birth (0.91-fold decrease in the LP group compared with the NP group, these data were presented as **Supplementary Materials** in an article previously published by our research group (Cavariani et al., 2019) (**Table 2**).

With regard to the other parameters analyzed on the day of offspring birth, this experimental model caused a significant reduction in crown-rump length (CRL) (0.97-fold reduction in the LP group compared with the NP group) and absolute anogenital distance (AGD) (0.87-fold reduction in the LP group compared with the NP group), as well as in relative AGD (0.9-fold reduction in the LP group compared with the NP group) (**Table 2**).

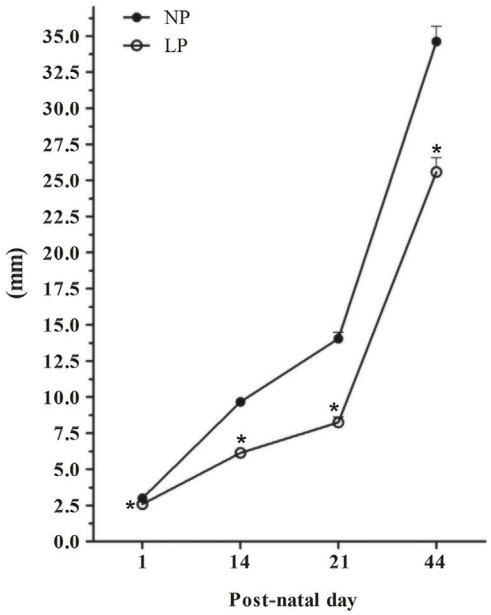

FIGURE 2 | Evolution of the anogenital distance. PND 1: NP, *n* = 17, LP, *n* = 19; PND 14: NP, *n* = 19, LP, *n* = 26; PND 21: NP, *n* = 17, LP, *n* = 22; PND 44: NP, *n* = 12, LP, *n* = 10. Evolution of AGD in animals whose mothers received normal-protein and low-protein diets during gestation and lactation. The values are expressed as the mean ± S.E.M. *$p < 0.05$. Student t-test was used to assess the significance of parametric data, and Mann-Whitney test was used to assess the significance in nonparametric data.

At PNDs 21 and 44, AGD remained significantly reduced in LP animals compared with NP animals (PND 21: 0.59-fold reduction in the LP group compared with the NP group; PND 44: 0.74-fold reduction in the LP group compared with the NP group) (**Figure 2**).

Although CRL remained significantly reduced in LP animals at PNDs 21 and 44 (PND 21: 0.67-fold reduction in the LP group compared with the NP group; PND 44: 0.8-fold reduction in the LP group compared with the NP group) (**Supplementary Table 1**), there was no significant difference in relative AGD between the groups at both ages (PND 21: 0.9-fold reduction in the LP group compared with the NP group; PND 44: 0.9-fold reduction in the LP group compared with the NP group) (**Supplementary Table 1**).

Regarding genital organ weight, the low-protein diet caused a significant decrease in the absolute weights of the testes and epididymides at all analyzed ages (testis weight: 0.41-fold decrease in the LP group compared with the NP group at PND 21; 0.55-fold decrease in the LP group compared with the NP group at PND 44; 0.79-fold decrease in the LP group compared with the NP group at PND 120. Epididymis weight: 0.52-fold decrease in the LP group compared with the NP group at PND 21; 0.51-fold decrease in the LP group compared with the NP group at PND 44; 0.84-fold decrease in the LP group compared with the NP group at PND 120) (**Table 3**).

Ventral prostate and empty seminal vesicle absolute weights were significantly reduced in LP animals at PNDs 21 and 44 (ventral prostate weight: 0.38-fold decrease in the LP group compared with the NP group at PND 21; 0.43-fold decrease in the LP group compared with the NP group at PND 44. Empty seminal vesicle weight: 0.42-fold decrease in the LP group compared with the NP group at PND 21; 0.40-fold decrease in the LP group compared with the NP group at PND 44) (**Table 3**), but there was no significant difference at PND 120 (0.83-fold decrease in ventral prostate weight in the LP group compared with the NP group; 1.12-fold increase in empty seminal vesicle weight in the LP group compared with the NP group) (**Table 3**).

The relative weights of genital organs were significantly elevated in LP animals at PND 21 (1.24-fold increase in relative testis weight in the LP group compared with the NP group; 1.62-fold increase in relative epididymis weight in the LP group compared with the NP group; 1.06-fold increase in relative ventral prostate weight in the LP group compared with the NP group; 1.30-fold increase in relative empty seminal vesicle weight in the LP group compared with the NP group) (**Table 3**).

At PND 44, the relative testis weight was slightly elevated in LP animals (1.02-fold increase in the LP group compared with the NP group) (**Table 3**), while for the epididymis and ventral prostate, the relative weights were slightly reduced in LP animals (0.95-fold decrease in relative epididymis weight in the LP group compared with the NP group; 0.78-fold decrease in relative ventral prostate weight in the LP group compared with

TABLE 3 | Body weight and absolute and relative genital organs weight at PND day 21, 44 and 120. PND 21: NP, n = 17, LP, n = 22; PND 44: NP, n = 12, LP, n = 10; PND 120: NP, n = 10, LP, n = 10. The values are expressed as the mean ± S.E.M. *$p < 0.05$. Student t-tests was used to analyze the significance of differences in parametric data ([a]), and Mann-Whitney tests was used to analyze the significance of differences in nonparametric data ([b]).

Parameters	PND 21		PND 44		PND 120	
	NP (*n* = 17)	LP (*n* = 22)	NP (*n* = 12)	LP (*n* = 10)	NP (*n* = 10)	LP (*n* = 10)
Body weight (g)	58.63 ± 1.59	19.29 ± 0.74[b*]	219.30 ± 4.99	122.50 ± 4.84[b*]	492.00 ± 13.96	399.90 ± 7.33[a*]
Testes (mg)	110.40 ± 2.79	45.30 ± 2.39 [b*]	944.10 ± 35.77	516.10 ± 27.03[a*]	1802 ± 68.05	1415 ± 39.23[a*]
Testes (mg/100 g)	189.50 ± 5.00	235.2 ± 8.80[a*]	412.30 ± 21.29	420.20 ± 12.65[b]	375.80 ± 14.87	355.30 ± 12.94[a]
Epididymis (mg)	17.29 ± 0.82	8.93 ± 0.40[b*]	141.70 ± 8.58	72.52 ± 4.27[a*]	826.40 ± 34.26	697.40 ± 21.20[a*]
Epididymis (mg/100 g)	28.65 ± 0.82	46.50 ± 1.62[b*]	61.98 ± 2.06	58.87 ± 1.67[a]	172.40 ± 7.87	175.40 ± 2.94[a]
Ventral prostate (mg)	21.49 ± 1.57	8.08 ± 0.59[b*]	103.70 ± 11.36	44.40 ± 5.70[a*]	928.50 ± 79.31	769.80 ± 63.45[a]
Ventral prostate (mg/100 g)	39.34 ± 3.42	41.67 ± 2.44[a]	46.85 ± 4.45	36.32 ± 4.42[a]	189.10 ± 12.97	190.70 ± 13.48[a]
Seminal vesicle (empty) (mg)	11.28 ± 0.38	4.70 ± 0.29[b*]	100.90 ± 10.94	39.31 ± 3.95[b*]	1472 ± 104.00	1647 ± 104.80[a]
Seminal vesicle (empty) (mg/100 g)	18.77 ± 0.88	24.46 ± 1.30[a*]	45.43 ± 4.34	31.80 ± 2.69[a*]	302.40 ± 27.27	424.40 ± 22.97[a*]

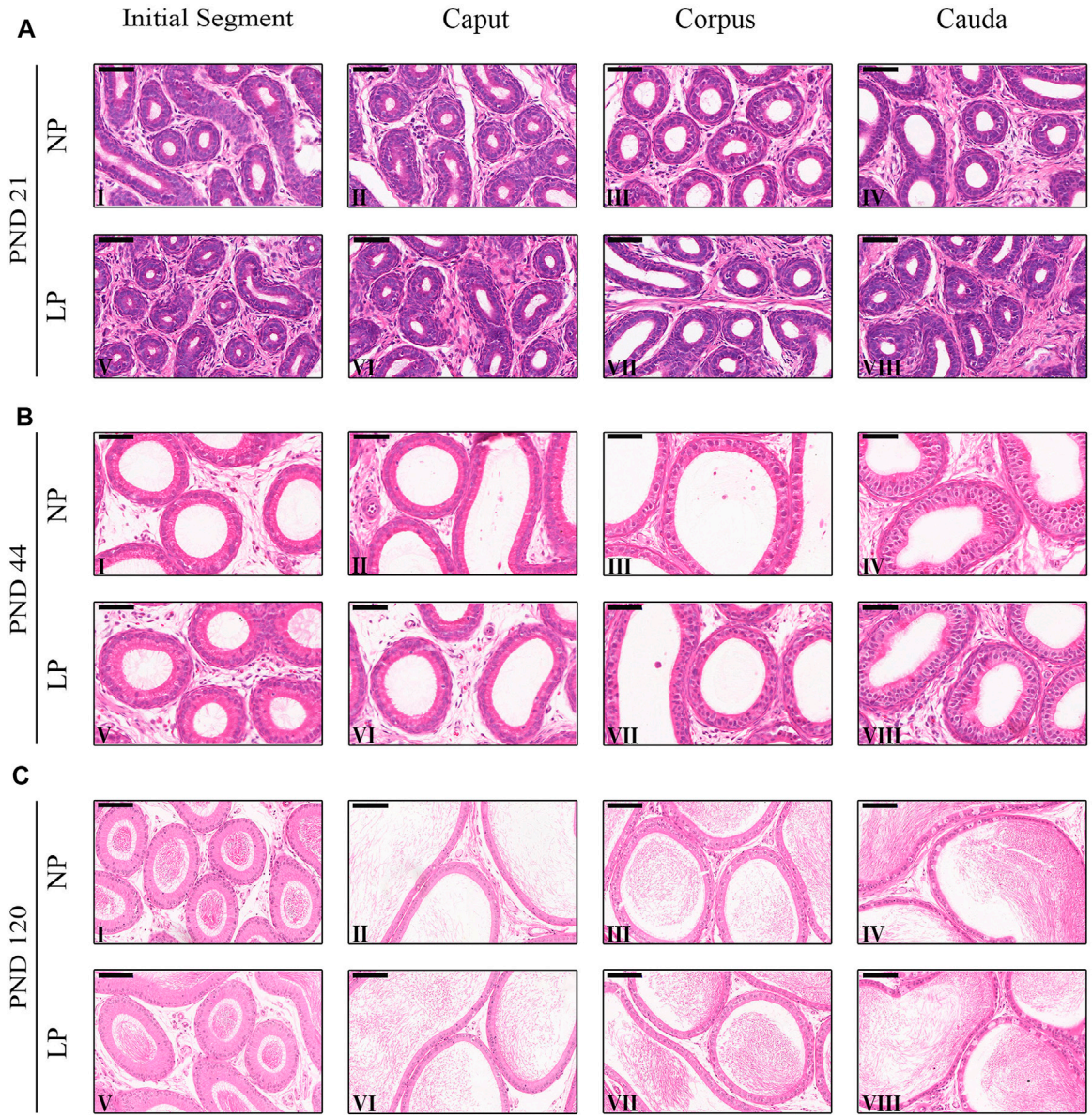

FIGURE 3 | Morphology of the epididymis. **(A)** Staining of the epididymides of NP and LP animals at PND 21 showing the initial segment **(I and V)**, caput **(III and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)**. H&E, Bar = 50 μm. **(B)** Staining of the epididymides of NP and LP animals at PND 44 showing the initial segment **(I and V)**, caput **(III and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** . H&E, Bar = 50 μm. **(C)** Staining of the epididymides of NP and LP animals at PND 120 showing the initial segment **(I and V)**, caput **(III and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)**. H&E, Bar = 100 μm.

the NP group) (**Table 3**). The relative empty seminal vesicle weight was significantly reduced in LP animals (0.70-fold decrease in the LP group compared with the NP group) (**Table 3**).

Finally, at PND 120, the relative weights of the epididymis and ventral prostate showed practically no differences between NP and LP animals (**Table 3**). The relative testis weight was slightly reduced in LP animals (0.95-fold decrease in the LP group compared with the NP group) (**Table 3**), and the relative empty seminal vesicle weight was significantly elevated in animals whose mothers received a low-protein diet (1.40-fold increase in the LP group compared with the NP group) (**Table 3**).

3.2 Maternal Protein Restriction did Not Change the Integrity of the Epididymal Epithelium or the Organ Interstitium but Changed the Tubular Diameter, Epithelial Height and Luminal Diameter of the Epididymal Duct

Both the NP and LP groups at PNDs 44 and 120 presented initial segment regions with small tubular diameters and organized epithelia containing principal cells, basal cells, narrow cells, and few apical cells (**Figures 3BI,V,CI,V**. The epididymal

TABLE 4 | – Morphometry of the epididymis. Tubular diameter, epithelium height and luminal diameter at PND 21, 44 and 120. NP, *n* = 4; LP, *n* = 4. Values expressed as means ± S.E.M. *$p < 0.05$. Student t-teste was used for parametric data (a), and Mann-whitney test was used for non-parametric data (b).

Parameters (μm)								
PND 21 (n = 4)	**Initial Segment**		**Caput**		**Corpus**		**Cauda**	
	NP	LP	NP	LP	NP	LP	NP	LP
Tubular diameter	40.88 ± 1.59	30.61 ± 0.45[a*]	48.34 ± 2.86	36.64 ± 0.85[a*]	60.60 ± 2.33	44.66 ± 1.45[a*]	58.57 ± 4.86	48.96 ± 4.15[a]
Epithelium height	12.22 ± 0.05	9.73 ± 0.19[b]	13.12 ± 0.78	10.93 ± 0.32[a*]	16.18 ± 0.63	12.27 ± 0.24[a*]	13.57 ± 1.09	14.15 ± 1.47[a]
Luminal diameter	17.68 ± 1.08	10.65 ± 0.29[a*]	21.99 ± 2.32	13.72 ± 0.12[b*]	26.81 ± 1.56	18.36 ± 1.54[a*]	34.45 ± 4.10	20.59 ± 2.34[a*]
***PND 44*(*n = 4*)**	**Initial Segment**		**Caput**		**Corpus**		**Cauda**	
	NP	***LP***	***NP***	***LP***	***NP***	***LP***	***NP***	***LP***
Tubular diameter	100.8 ± 4.36	97.91 ± 0.78[a]	131.3 ± 12.69	114.1 ± 7.63[a]	188.1 ± 14.56	147.0 ± 12.50[a]	118.5 ± 2.49	113.1 ± 3.82[a]
Epithelium height	19.44 ± 0.33	19.96 ± 0.47[a]	15.67 ± 0.67	15.86 ± 0.68[a]	17.91 ± 1.42	20.01 ± 1.50[a]	24.63 ± 0.59	22.78 ± 0.25[*a]
Luminal diameter	61.54 ± 4.63	58.51 ± 0.99[a]	99.11 ± 14.26	84.58 ± 7.41[a]	152.6 ± 17.42	108.1 ± 16.98[a]	64.97 ± 1.46	63.94 ± 3.38[a]
***PND 120*(n = *4*)**	**Initial Segment**		**Caput**		**Corpus**		**Cauda**	
	NP	***LP***	***NP***	***LP***	***NP***	***LP***	***NP***	***LP***
Tubular diameter	172.8 ± 11.26	173.9 ± 7.68[a]	320.5 ± 23.68	318.9 ± 8.31[a]	326.1 ± 8.95	298.4 ± 11.37[a]	289,7 ± 6,12	312,3 ± 19,11[b]
Epithelium height	29.54 ± 0.40	33.38 ± 3.72[a]	22,07 ± 1,31	21,63 ± 0,01[b]	23.02 ± 1.12	23.95 ± 1.15[a]	19.13 ± 1.23	18.85 ± 1.53[a]
Luminal diameter	107.5 ± 5.52	99.77 ± 6.55[a]	276.6 ± 21.32	273.3 ± 9.77[a]	280.2 ± 10.65	252.2 ± 10.54[a]	239.8 ± 10.86	274.4 ± 21.86[a]

caput also had a well-organized epithelium with the presence of principal cells, basal cells and clear cells. In this region, the tubular diameter was slightly greater than that in the initial segment region (**Figures 3BII,VI,CII,VI**). With age advancement, the epididymal corpus showed principal cells with slightly more cubic shapes than the principal cells of the initial segment and caput regions in addition to presenting clear cells and basal cells (**Figures 3BIII,VII,CIII,VII**). In the cauda region, the epithelial height did not differ greatly from the epithelial height of the caput region between the groups of animals at 21 and 44 days but was much reduced in the animals at 120 days. On PNDs 44 and 120, principal cells, basal cells and a greater number of clear cells were observed in the caudal region compared to the other epididymal regions (**Figures 3BIV,VIII,CIV,VIII**).

LP animals at PND 21 presented reductions in tubular diameter, epithelial height and luminal diameter in all epididymal regions. However, the decrease in tubular diameter in the cauda and the decreases in epithelial height in the initial segment and cauda were not significant at this age (**Table 4**).

At PND 44, the tubular diameter and luminal diameter were decreased nonsignificantly in all epididymal regions of animals whose mothers received a low-protein diet during gestation and lactation. Conversely, the LP animals presented a slight increase in epithelial height in the initial segment, caput and corpus, while in the cauda region, a significant decrease in epithelial height was observed (**Table 4**).

In 120-day-old animals, maternal protein restriction had slightly decreased the tubular diameter in the caput and corpus regions and slightly increased this parameter in the initial segment and cauda regions. Conversely, epithelial height was increased nonsignificantly in the caput and corpus regions but decreased nonsignificantly in the initial segment and cauda region. Finally, at this age, the luminal diameter was decreased nonsignificantly in all epididymal regions of LP animals, except for the cauda region, in which a slight increase in luminal diameter was observed (**Table 4**).

3.3 Maternal Protein Restriction did Not Alter Estradiol Serum Levels in Male Offspring

In 21-day-old animals, only a slight increase in circulating estradiol was observed in rats whose mothers received a low-protein diet (1.90-fold increase in the LP group compared with the NP group). At PND 120, LP animals showed a slight decrease in this steroid hormone (0.87-fold reduction in the LP group compared with the NP group) (data not shown).

It was not possible to measure serum estradiol levels in 44-day-old animals because the values of this steroid hormone were below the lower limit of detection of the chemiluminescence technique (0.017 ng/dl). This occurred for both LP and NP animals, impeding comparison between the groups.

3.4 Impact of the Maternal Low-Protein Diet on AR, ERα and ERβ Immunolocalization

Immunolocalization of AR, ERα and ERβ was observed in the nuclei of epididymal epithelial cells and in epididymal interstitial cells at all analyzed ages. Furthermore, in the 44- and 120-day-old animals, these receptors were also labeled in peritubular smooth muscle cells.

3.4.1 AR Immunolocalization

In both groups at PND 21, the AR-labeling pattern appeared more intense and uniform in epididymal epithelial cells, whereas in mesenchymal cells, it was less intense and more heterogeneous; these findings are consistent with those of previous studies investigating the immunolocalization of AR in the epididymides of young rats (You and Sar, 1998; Zaya et al., 2012). However, the AR-labeling intensity was slightly increased in the epididymal caput and cauda in LP animals of this age compared to NP animals of this age (**Figure 4A** LP VI and VIII; NP II and IV).

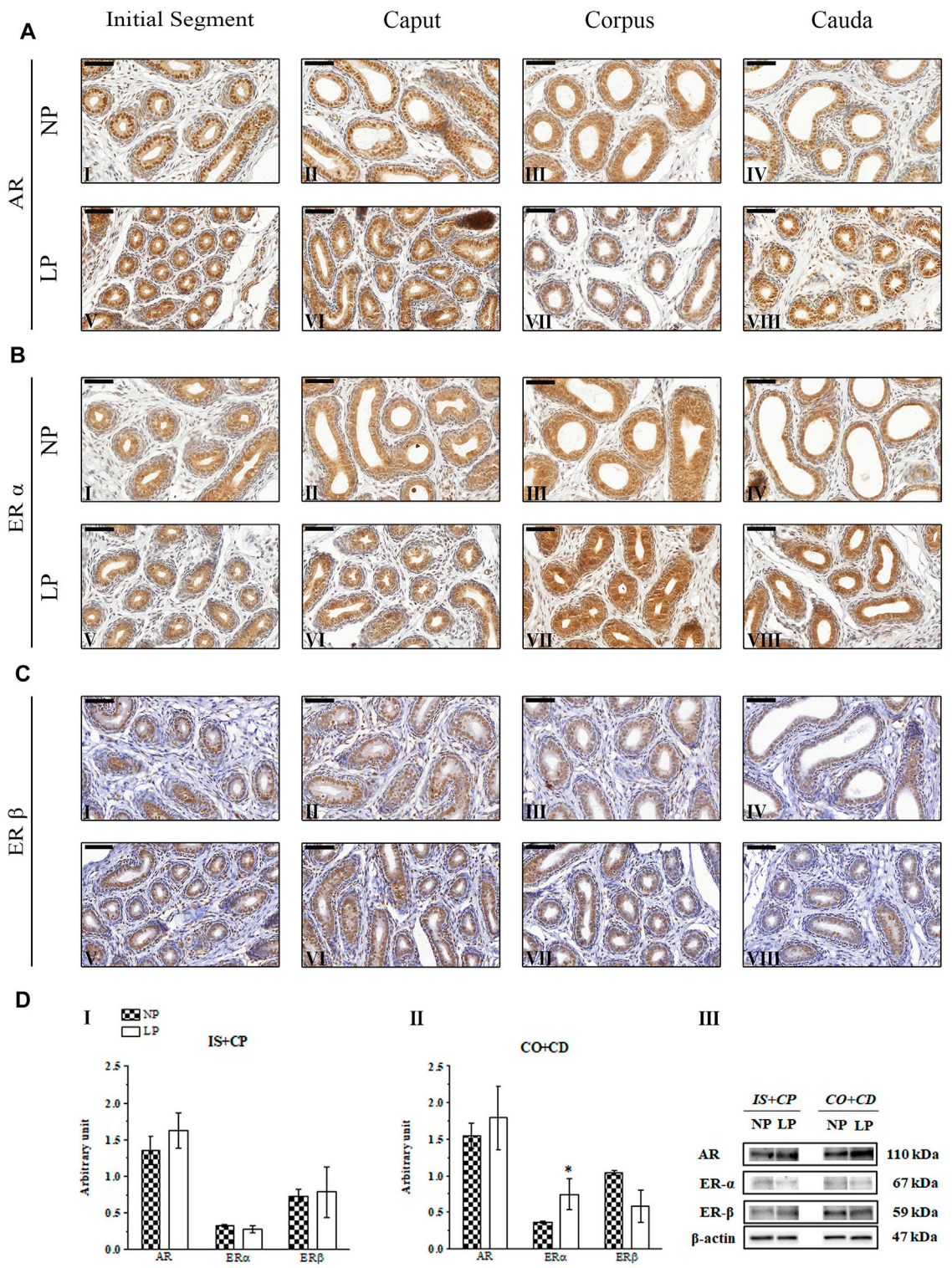

FIGURE 4 | Expression and immunolocalization of AR, ERα and ERβ in the epididymides of 21-day-old animals. **(A)** Immunoreactivity for AR in epithelial cells and in mesenchymal cells in the initial segment (**I and V**), caput (**II and VI**), corpus (**III and VII**) and cauda (**IV and VIII**) regions of NP and LP animals. **(B)** Immunoreactivity for ERα in epithelial cells and in mesenchymal cells in the initial segment (**I and V**), caput (**II and VI**), corpus (**III and VII**) and cauda (**IV and VIII**) regions of NP and LP animals. **(C)** Immunoreactivity for ERβ in epithelial cells and in mesenchymal cells in the initial segment (**I and V**), caput (**II and VI**), corpus (**III and VII**) and cauda (**IV and VIII**) regions of NP and LP animals. Bar = 50 μm. **(D)** Extracts obtained from individual animals were used for densitometric analysis of the levels of the proteins in the initial segment plus caput (**I**) and the corpus plus cauda (**II**) regions following normalization to the levels of the housekeeping protein β-actin. The representative blots show the protein levels of AR, ERα, ERβ and β-actin (**III**, right panel). The data are presented as the mean ± S.E.M. *$p < 0.05$, Mann-Whitney test.

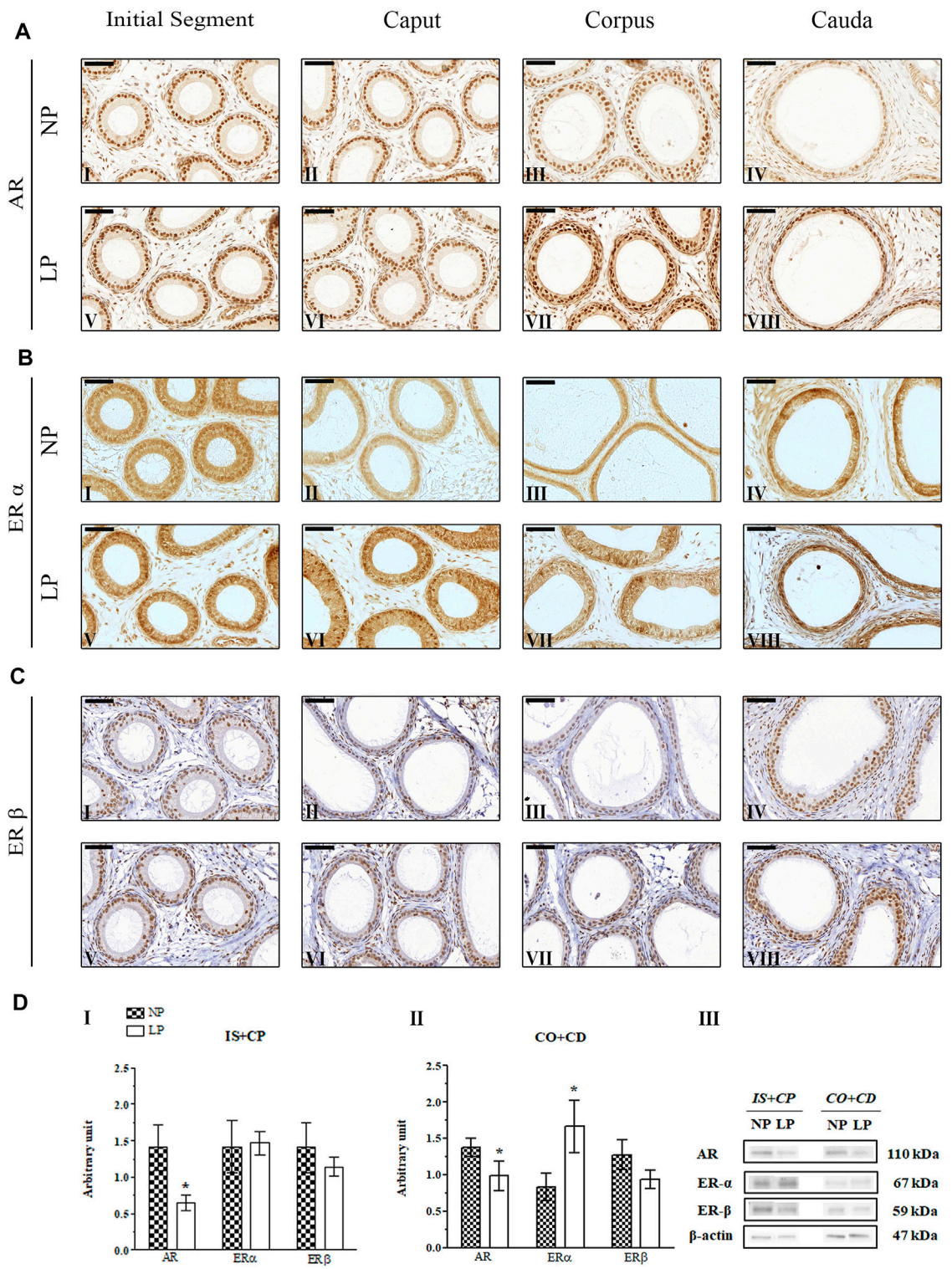

FIGURE 5 | Expression and immunolocalization of AR, ERα and ERβ in the epididymides of 44-day-old animals**. (A)** Immunoreactivity for AR in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(B)** Immunoreactivity for ERα in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(C)** Immunoreactivity for ERβ in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. Bar = 50 μm. **(D)** Extracts obtained from individual animals were used for densitometric analysis of the levels of the proteins in the initial segment plus caput **(I)** and corpus plus cauda **(II)** regions following normalization to the levels of the housekeeping protein β-actin. The representative blots show the protein levels of AR, ERα, ERβ and β-actin (**III**, right panel). The data are presented as the mean ± S.E.M. *$p < 0.05$, Mann-Whitney test.

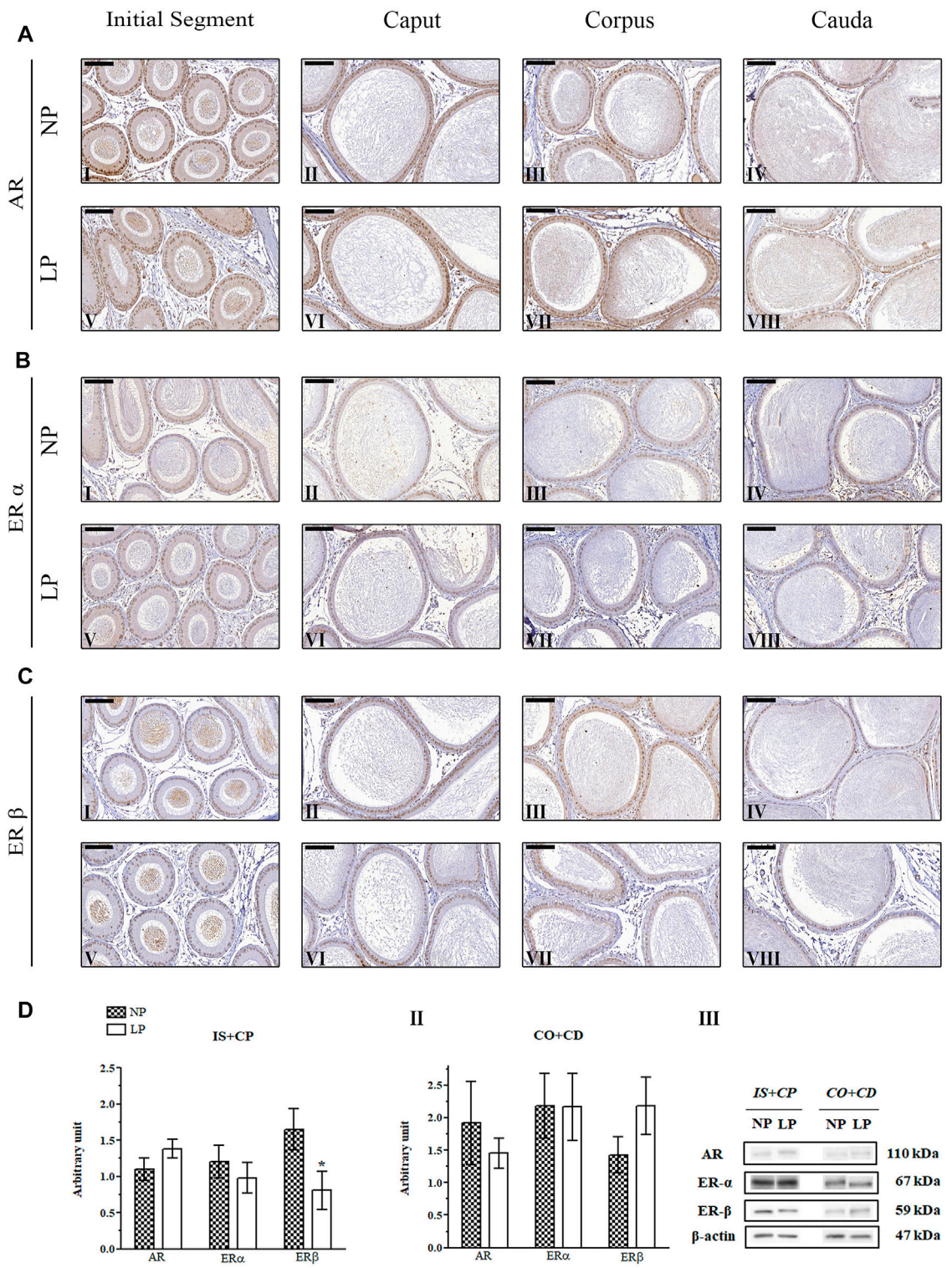

FIGURE 6 | Expression and immunolocalization of AR, ERα and ERβ in the epididymides of 120-day-old animals. **(A)** Immunoreactivity for AR in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(B)** Immunoreactivity for ERα in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. **(C)** Immunoreactivity for ERβ in epithelial cells and in mesenchymal cells in the initial segment **(I and V)**, caput **(II and VI)**, corpus **(III and VII)** and cauda **(IV and VIII)** regions of NP and LP animals. Bar = 100 μm. **(D)** Extracts obtained from individual animals were used for densitometric analysis of the levels of the proteins in the initial segment plus caput **(I)** and corpus plus cauda **(II)** regions following normalization to the levels of the housekeeping protein β-actin. The representative blots show the protein levels of AR, ERα, ERβ and β-actin (**III**, right panel). The data are presented as the mean ± S.E.M. $^{*}p < 0.05$, Mann-Whitney test.

In the 44-day-old NP animals, AR immunolocalization was observed in the nuclei of epididymal epithelial cells, with slightly more intense staining in the caput and cauda regions than in the other regions of the epididymis, corroborating the findings of Yamashita (2004), Perobelli et al. (2013) and Leite et al. (2014) (**Figures 5AI,IV**). Although we observed significant reductions in AR expression in the epididymal IS + CP and CO + CD regions of LP animals at PND 44 (**Figures 5CI,II**), there were no differences in the labeling pattern for this receptor between the NP and LP groups at this age (**Figure 5A**).

In the epididymal epithelia of 120-day-old NP and LP animals, the nuclear staining of AR was more intense and homogeneous in the principal cells than in the other cells throughout the organ (**Figures 6AI–VIII**). Clear cells of the cauda region showed quite heterogeneous staining ranging from very discrete nuclear labeling to absent labeling in these cells (Paris et al., 1994; Zhu et al., 2000; Kaushik et al., 2010; Zaya et al., 2012) (**Figures 6AIV,VIII**).

3.4.2 ERα Immunolocalization

At PNDs 21 and 44, NP animals showed mesenchymal cells with ERα labeling that was heterogeneous, moderate and only nuclear, while in the epithelial cells, the staining for this receptor appeared in a homogeneous way in the nucleus and cytoplasm throughout the epididymis. In differentiated clear cells, ERα labeling was only nuclear (Zaya et al., 2012) (**Figures 4BI–IV**; **Figures 5BI–IV**). The ERα immunolocalization pattern observed in 21- and 44-day-old LP rats did not differ from that observed for NP rats at these ages. However, the intensity of cytoplasmic labeling in the cauda region was more intense in LP animals than in NP animals of both ages, staining even the clear cell cytoplasm of this region (**Figure 4BVIII**; **Figure 5BVIII**).

No differences were observed in the ERα immunolocalization pattern between NP and LP animals at PND 120. In both groups, nuclear and cytoplasmic ERα labeling was observed in epididymal epithelial cells, mainly in principal cells, in all regions of the organ (Hess et al., 1997; Kolasa et al., 2003). Nuclear labeling for this receptor was also observed in interstitial and peritubular smooth muscle cells throughout all regions of the epididymis (Hess et al., 1997; Hess et al., 2011; Zaya et al., 2012) (**Figures 6BI–VIII**).

3.4.3 ERβ Immunolocalization

The same pattern of ERβ immunolocalization was observed for NP and LP animals at PND 21. The intensity of nuclear and cytoplasmic ERβ labeling in epididymal epithelial cells varied considerably, and some of these cells showed a complete absence of immunoreactivity. A decreasing gradient was observed in the nuclear ERβ staining intensity from the initial segment and caput to the corpus and cauda of the organ. Immunostaining was also observed in the mesenchymal cells surrounding epididymal ducts (Sar and Welsch, 2000; Zaya et al., 2012) (**Figures 4CI–VIII**).

There was no difference in the pattern of ERβ labeling in LP animals compared to NP animals at PND 44. In both groups, epithelial cells, mainly principal cells, showed homogeneous nuclear immunostaining and discrete cytoplasmic labeling throughout the epididymis, with elevated nuclear staining intensity in the cauda region. In addition, heterogeneous nuclear labeling was observed in mesenchymal and peritubular smooth muscle cells in all regions of the organ (**Figures 5CI–VIII**).

The 120-day-old NP and LP animals showed the same pattern of ERβ immunostaining. Nuclear and cytoplasmic labeling of this hormone receptor was observed in epithelial cells throughout the epididymis. Principal cells presented homogeneous nuclear staining, while the other epididymal epithelial cells, peritubular smooth muscle cells and interstitial cells showed heterogeneous labeling (Choi et al., 2001; Kolasa et al., 2003; Yamashita, 2004; Zaya et al., 2012) (**Figures 6I–VIII**). Nuclear labeling of epithelial cells in the caput region was weak in LP animals compared to NP animals (**Figures 6CIV,VIII**).

3.5 Maternal Low-Protein Diet Changes AR, ERα and ERβ Expression in the Epididymides of Offspring in an Age-dependent Manner

A low-protein diet during gestation and lactation significantly decreased AR expression in the IS + CP (0.50-fold decrease in the LP group compared with the NP group) and CO + CD (0.72-fold decrease in the LP group compared with the NP group) at PND 44 (**Figures 5DI,II**). In 21-day-old animals, maternal protein restriction slightly increased AR expression in the IS + CP (1.20-fold increase in the LP group compared with the NP group) and CO + CD (1.17-fold increase in the LP group compared with the NP group) (**Figures 4DI,II**). In addition, at PND 120, this experimental model resulted in a nonsignificant reduction in the AR levels in the CO + CD region of the epididymis (0.76-fold decrease in the LP group compared with the NP group) (**Figures 6DII**).

The low-protein diet significantly increased ERα expression in the CO + CD region at PNDs 21 and 44 (PND 21: 2.10-fold decrease in the LP group compared with the NP group; PND 44: 2.01-fold decrease in the LP group compared with the NP group) (**Figure 4DII**; **Figure 5DII**). ERα expression was only slightly reduced in the IS + CP region of LP animals at PND 21 (0.85-fold decrease in the LP group compared with the NP group) and in the IS + CP and CO + CD regions of LP animals at PND 120 (IS + CP: 0.82-fold decrease in the LP group compared with the NP group; CO + CD: 0.99-fold decrease in the LP group compared with the NP group) (**Figure 4D Figures 6DI,II**).

There was no difference in ERβ expression between NP and LP animals at PNDs 21 and 44. Only in 120 day-old animals was a significant decrease in ERβ expression observed in the IS + CP region (0.50-fold decrease in the LP group compared with the NP group) (**Figures 6DI**).

3.6 Aromatase p450, but Not 5α-Reductase, Expression Is Altered by Maternal Protein Restriction

A recently published study by our research group showed that maternal protein restriction during gestation and lactation significantly decreased serum testosterone levels in 44-day-old animals (Cavariani et al., 2019). However, the epididymal expression of 5α-reductase was unchanged at all of the analyzed

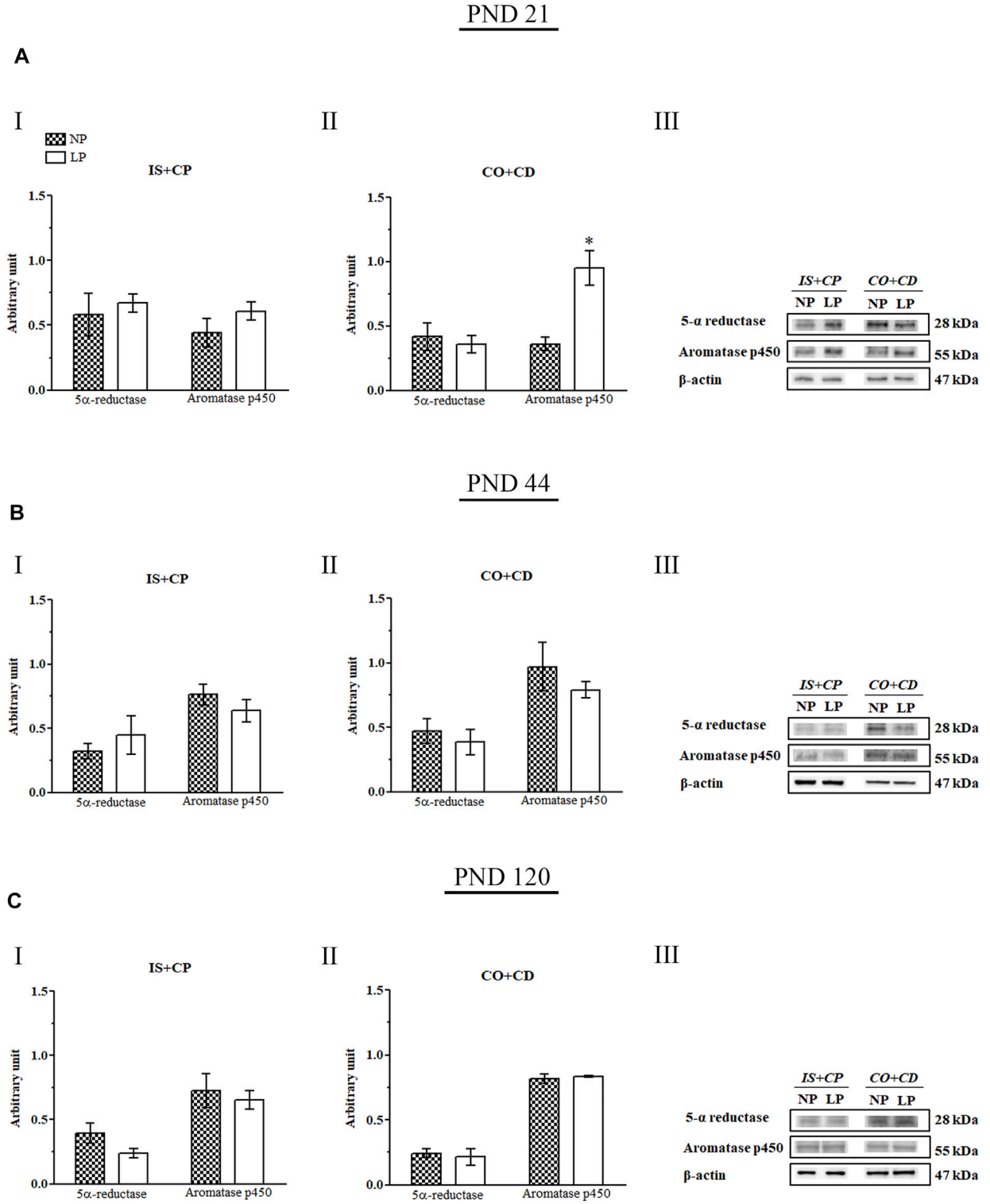

FIGURE 7 | Immunoblots of 5α-reductase and aromatase p450. **(A)** Levels of 5α-reductase and aromatase p450 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 21. The representative blots show the protein levels of 5α-reductase, aromatase p450, and β-actin (70 μg of protein) in 21-day-old animals **(III)**. **(B)** Levels of 5α-reductase and aromatase p450 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 44. The representative blots show the protein levels of 5α-reductase, aromatase p450 and β-actin (70 μg of protein) in 44-day-old animals **(III)**. **(C)** Levels of 5α-reductase and aromatase p450 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 120. The representative blots show the protein levels of 5α-reductase, aromatase p450 and β-actin (70 μg of protein) in 120-day-old animals **(III)**. The data are presented as the mean ± S.E.M. $^*p < 0.05$, Mann-Whitney test.

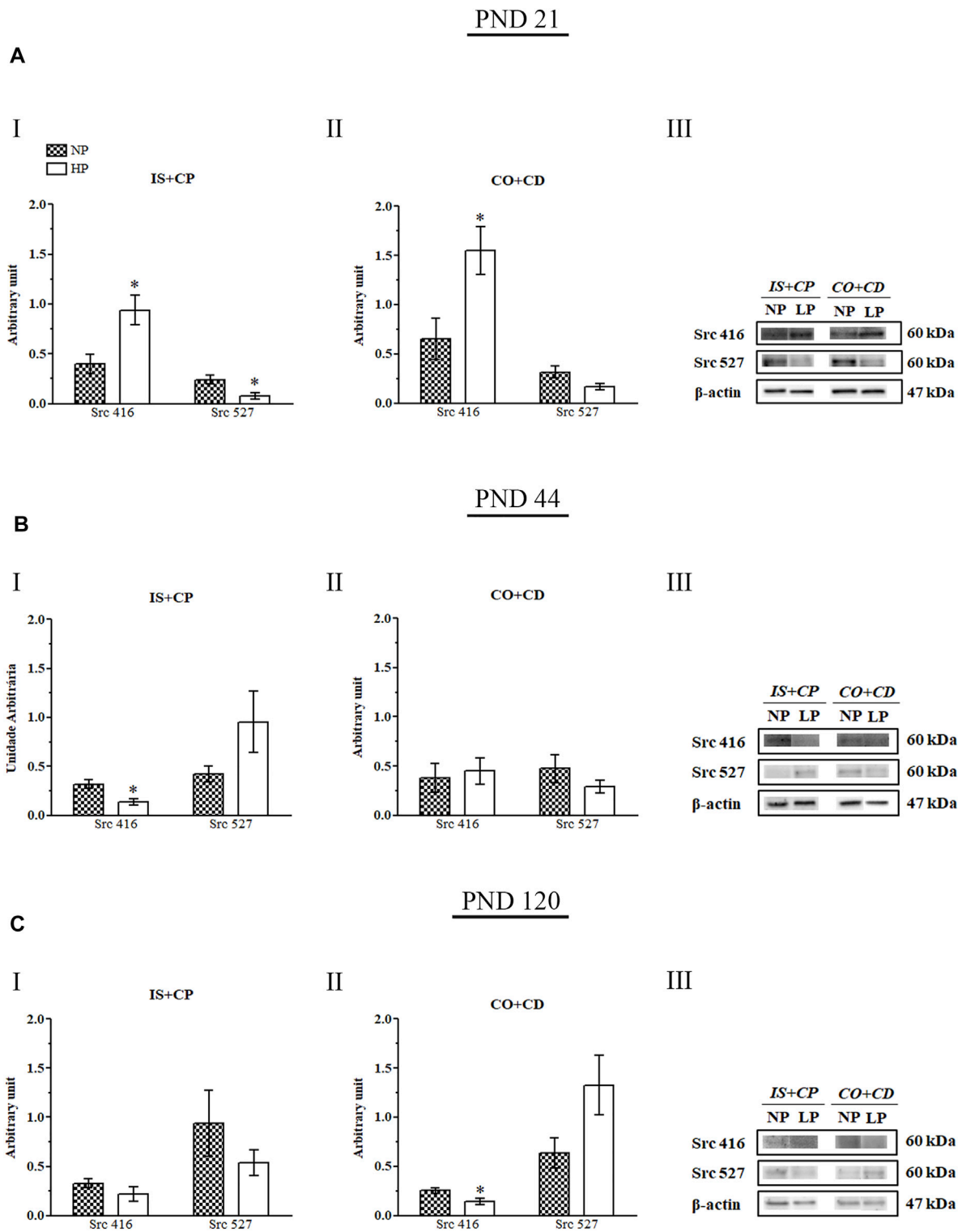

FIGURE 8 | Immunoblots of Src 416 and Src 527. **(A)** Levels of Src 416 and Src 527 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 21. The representative blots show the protein levels of Src 416, Src 527 and β-actin (70 μg of protein) in 21-day-old animals **(III)**. **(B)** Levels of Src 416 and Src 527 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 44. The representative blots show the protein levels of Src 416, Src 527 and β-actin (70 μg of protein) in 44-day-old animals **(III)**. **(C)** Levels of Src 416 and Src 527 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 120. The representative blots show the protein levels of Src 416, Src 527 and β-actin (70 μg of protein) in 120-day-old animals **(III)**. The data are presented as the mean ± S.E.M. *$p < 0.05$, Mann-Whitney test.

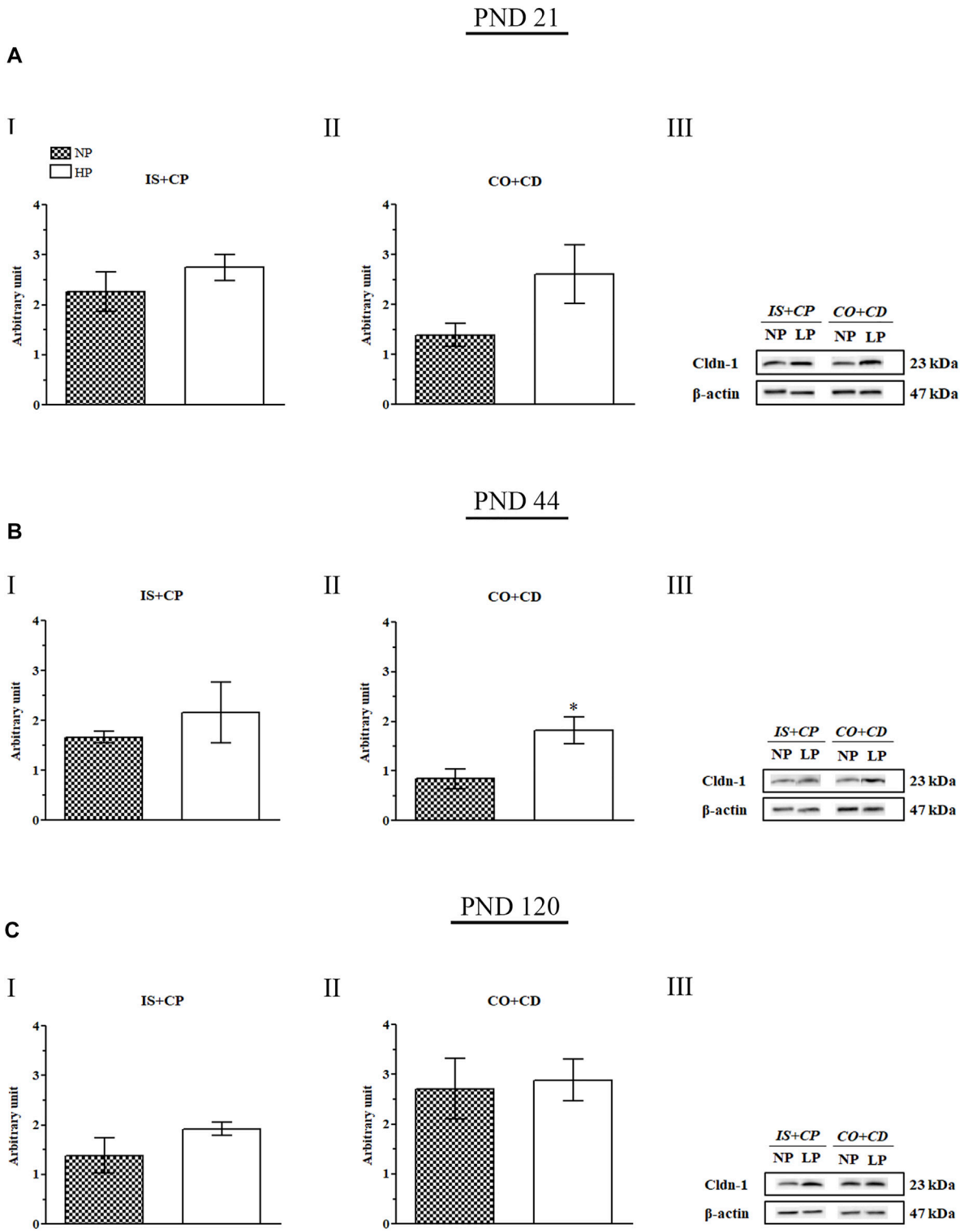

FIGURE 9 | Cldn-1 immunoblot. **(A)** Levels of Cldn-1 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 21. The representative blots show the protein levels of Cldn-1 and β-actin (70 μg of protein) in 21-day-old animals **(III)**. **(B)** Levels of Cldn-1 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 44. The representative blots show the protein levels of Cldn-1 and β-actin (70 μg of protein) in 44-day-old animals **(III)**. **(C)** Levels of Cldn-1 in the IS + CP **(I)** and CO + CD **(II)** epididymal regions of NP and LP animals on PND 120. The representative blots show the protein levels of Cldn-1 and β-actin (70 μg of protein) in 120-day-old animals **(III)**. The data are presented as the mean ± S.E.M. $^{*}p < 0.05$, Mann-Whitney test.

ages (**Figure 7**). Notably, we observed a significant increase in aromatase p450 expression in the CO + CD region of the epididymis in PND 21 animals (2.64-fold increase in the LP group compared with the NP group) (**Figures 7AII**).

3.7 Maternal Protein Restriction Changed Both Src 416 and Src 527 Expression in an Age-dependent Manner

The low-protein diet increased Src 416 expression in the IS + CP (2.35-fold increase in the LP group compared with the NP group) and CO + CD (2.38-fold increase in the LP group compared with the NP group) regions of 21-day-old animals. In addition, at this same age, the Src 416 increase appeared to be accompanied by a decrease in Src 527 expression in the IS + CP region (0.33-fold decrease in the LP group compared with the NP group) in LP animals (**Figures 8AI,II**).

At PND 44, LP animals presented a significant decrease in Src 416 expression in the IS + CP region (0.44-fold decrease in the LP group compared with the NP group), while Src 527 expression was increased nonsignificantly in this region (2.26-fold increase in the LP group compared with the NP group) (**Figure 8BII**).

In adulthood (PND 120), we also observed a significant decrease in Src 416 expression accompanied by a nonsignificant increase in Src 527 expression in animals whose mothers received a low-protein diet during gestation and lactation (0.56-fold decrease in Src 416 levels in the LP group compared with the NP group; 2.06-fold increase in Src 527 levels in the LP group compared with the NP group). However, for PND 120, these results were observed in the CO + CD epididymal region of LP rats (**Figures 8CII**).

3.8. The Low-Protein Diet Increased Cldn-1 Expression Throughout the Epididymis

Maternal protein restriction during gestation and lactation increased Cldn-1 expression in the IS + CP and CO + CD epididymal regions at all ages analyzed. However, these results were only significant for Cldn-1 in the CO + CP region (2.17-fold increase in the LP group compared with the NP group) on PND 44. At this age, Cldn-1 expression was only slightly increased in the IS + CP region (1.3-fold decrease in the LP group compared with the NP group) (**Figures 9BI,II**).

Importantly, although the difference was not significant, Cldn-1 expression was increased in the IS + CP (1.22-fold increase in the LP group compared with the NP group) and CO + CD (1.88-fold increase in the LP group compared with the NP group) regions at PND 21 (**Figures 9AI,II**). This same pattern of Cldn-1 expression was observed in 120-day-old animals, both in the IS + CP (1.39-fold increase in the LP group compared with the NP group) and CO + CD (1.06-fold increase in the LP group compared with the NP group) regions (**Figures 9CI,II**).

4 DISCUSSION

Low birth weight is an important sign of malnutrition during pregnancy and a crucial indicator of slow fetal growth (Jahan-Mihan et al., 2015). Adverse intrauterine nutritional conditions are able to program a series of adaptations in the developing fetus constituting an "economic" phenotype in order to increase the chances of immediate survival of the fetus and to grant advantages in a postnatal environment of nutritional scarcity (Hales and Barker, 1992). Therefore, the fetus interacts with the maternal environment dynamically in an attempt to predict the environment in which it is likely to be born, adapting for future competitive advantage (Gluckman and Hanson, 2006).

Maternal protein restriction is able to promote growth restriction at birth followed by the subsequent catch-up growth (Ozanne and Hales, 2004; Ozanne and Nicholas Hales, 2005). However, several studies with pregnant rats fed a low-protein diet reported pups with reduced body weight at birth which was maintained up to one year of ag (Zeman, 1967; Zambrano et al., 2005; Hoppe et al., 2007; Fetoui et al., 2009; Qasem et al., 2016; Yuasa et al., 2016). Consistent with these results, we observed lower body weight of the male offspring at birth after maternal protein restriction and at all analyzed ages. Our results showed that the components and quality of the maternal diet during critical periods of development may alter the development of offspring in the uterus and modify their phenotypes in adulthood.

Some studies have shown that maternal protein restriction does not alter offspring weight at birth, whereas others have demonstrated that insufficient protein intake during gestation and lactation significantly reduces the birth weights of both male and female pups (Burdge et al., 2003; Fernandez-Twinn et al., 2003; Torres et al., 2010; Whitaker et al., 2012; Rebelato et al., 2013; Claycombe et al., 2015; Gonzalez et al., 2016; Yuasa et al., 2016). The lower birth weight observed in the offspring whose mothers were fed a low-protein diet could be related to adaptation to a protein-deficient intrauterine environment, preparing the pups' bodies to survive in a postnatal environment where the protein supply would also be low. Furthermore, during pregnancy, increased protein intake is recommended to attend the additional demand for nitrogen required by both mother and fetus (Jolly et al., 2004). With reduced protein intake, the pregnant rats of the LP group could not provide their developing offspring enough protein to reach a size similar to that of the offspring of rats fed a normal-protein diet. The CRLs of LP pups were also significantly lower than the CRLs of NP pups, consistent with their lower birth weights and with the results of other studies using this experimental model (Zeman, 1967; Colombelli et al., 2017).

AGD is a marker of sexual differentiation that reflects the action of androgenic hormones during the formation of the genital system in the uterus, being on average twice as large in males as in females (Graham and Gandelman, 1986; Welsh et al., 2008; Kita et al., 2016). Anogenital distance is usually regulated by testosterone produced by fetal testicles and is also affected by maternal androgens via the placenta (Graham and Gandelman, 1986). However, AGD reduction in LP pups could be correlated to the size of the pup independently of its intrauterine environment, since lighter and smaller animals tend to have significantly shorter AGDs than larger animals (Graham and Gandelman, 1986; Kerin et al., 2003; Dusek et al., 2010).

The difference in AGD and in CRL between NP and LP animals was maintained until PND 44, showing that maternal protein restriction during gestation and lactation delays the development of male offspring. However, when we observed the value of the AGD normalized by the CRL value, this difference no longer appear, indicating that at PNDs 21 and 44, the increase in AGD was proportional to the increase in CRL in LP animals, causing the ratio of these values to approach that of the values found for NP animals.

There is a maximum potential growth of anogenital distance programmed *in utero* (Scott et al., 2007; Kita et al., 2016). In male rats, AGD lengthens until PND 38, remaining constant from that period, and responds to hormonal stimuli during pubertal development, being negatively modulated by high doses of antiandrogens and testosterone (Kita et al., 2016). Thus, the maintenance of AGD reduction found in LP animals could be a consequence of protein restriction-induced programming during intrauterine life in addition to being a response to the slight increase in serum testosterone levels observed in 21-day-old animals. The decrease in AGD was maintained until PND 44, even with the significant reductions in testosterone levels observed in LP animals at this age [data related to testosterone levels are included in a recently published study from our research group Cavariani et al. (2019)].

Few epidemiological and experimental studies have addressed the effects of maternal protein restriction on offspring reproductive aspects, especially male reproductive aspects. Regarding the weight of genital system organs, the literature is controversial; studies have yielded results ranging from reductions in this parameter to no alterations in this parameter in animals whose mothers received a low-protein diet during gestation and lactation (Zambrano et al., 2005; Toledo et al., 2011; Rodriguez-Gonzalez et al., 2012; Rodriguez-Gonzalez et al., 2014).

The testes are the organs where gamete production occurs, while the epididymides are responsible for the storage, protection, concentration and maturation of these gametes. Therefore, the survival of mammals depends on these organs being fully functional (Cosentino and Cockett, 1986; Hermo and Robaire, 2002; Gatti et al., 2004). As previously mentioned, some effects of protein restriction are direct consequences of the alteration in the availability of substrate, and during pregnancy, increased protein intake is recommended to supply the requirements of rapid embryo growth (Godfrey and Barker, 2000; Jolly et al., 2004). In this context, the increased relative weight of these organs in 21-day-old LP animals could be an attempt to preserve their full functionality despite the poor nutritional environment to which the animals were exposed during their development and early postnatal life. However, even though the epididymal weight in relation to body weight was higher in LP animals at this age, the epididymal morphometry showed that the diameter of the epididymal duct, the diameter of the epididymal lumen, and the height of the epithelium were significantly smaller in LP animals compared to NP animals.

According to the "thrifty phenotype hypothesis", adverse intrauterine nutritional conditions are able to program adaptations in the developing fetus to increase its chances of immediate survival and to confer advantages for its long-term survival in a postnatal environment of nutritional scarcity (Hales and Barker, 1992). The phenotype of an organism will tend to be normal if there is similarity between the pre- and postnatal environments. However, if the postnatal environment is incompatible with the predicted environment, fetal programming will make the organism susceptible to metabolic diseases (Armitage et al., 2005; Martin-Gronert and Ozanne, 2010; Qasem et al., 2012). Indeed, several studies have shown that a maternal low-protein diet during gestation and lactation can lead to permanent metabolic changes in offspring, even if the offspring have access to normal-protein diets after weaning (Zambrano et al., 2005; Zambrano et al., 2006; Colombelli et al., 2017; Santos et al., 2018; Cavariani et al., 2019). In the current study, animals from both groups were fed a normal-protein diet after weaning (PND 21). The increase in protein intake by the animals of the LP group seems to have been enough to increase the weight of these animals in relation to the weight of their organs and to increase epididymal diameter and epithelial height, since the increases in the relative weights of the testes, epididymides, and seminal glands and the decreases in epididymal morphometry observed for LP animals at PND 21 no longer appeared in these animals at PNDs 44 and 120. However, we cannot affirm that this restoration was enough to prevent changes in the functions of these organs.

At 44 days, the absolute and relative weights of the empty seminal glands were lower in LP animals than in NP animals. As these organs are very sensitive to testosterone, these decreases could be directly related to the lower concentrations of this hormone observed in these animals (Cavariani et al., 2019). Rats at PND 120 are sexually mature, and their seminal glands are full of a fluid that contributes to the coagulation of ejaculated semen (Mann, 1974). The increase in empty seminal gland weight in 120-day-old LP animals could have been due to a higher concentration of this fluid in animals of this group, independent of their seminal gland size.

AR is a member of the steroid receptor superfamily that plays a key role in the action of androgenic hormones (Chang et al., 1995). Increases in testosterone levels appear to be accompanied by increased expression of AR (Bentvelsen et al., 1995; Wang et al., 2016) while decreases in testosterone levels are followed by reductions in the expression of this hormone receptor (Zhu et al., 2000; Liu and Wang, 2005). Thus, the nonsignificant increases in the expression of AR in the epididymal IS + CP and CO + CD regions observed in 21-day-old LP animals could have been a consequence of a nonsignificant increase in serum testosterone levels in these animals (Cavariani et al., 2019). Similarly, the reductions in AR expression in both the IS + CP and the CO + CD regions of the LP animal epididymides at PND 44 could have been related to the decreases in the levels of this androgenic hormone observed in these animals (Cavariani et al., 2019).

In the 120-day-old LP animals, the nonsignificant decrease in serum testosterone levels (Cavariani et al., 2019) appeared to be accompanied by a nonsignificant decrease in AR expression in the CO + CD region, consistent with the results obtained for the LP animals at PND 44. However, in the epididymal IS + CP regions of these animals, the AR expression was slightly increased,

suggesting possible nonandrogenic regulation of this receptor. Although several studies have demonstrated a positive effect of testosterone on AR expression, the mechanisms by which this regulation occurs are still not completely understood (Bentvelsen et al., 1995; Zhu et al., 2000; Ezer, 2002; Heinlein and Chang, 2002; Takayama, 2017). Furthermore, in addition to testosterone, AR can be transactivated by Src kinase through Tyr 543 phosphorylation, thereby triggering an extensive set of AR-dependent genes (Guo et al., 2006; Chattopadhyay et al., 2017). Moreover, prostate samples of men with castration-resistant prostate cancer present increased Src pathway activity in tumors with low AR activity, suggesting that Src activity probably has a strong negative correlation with AR activity (Mendiratta et al., 2009). The slight increase in AR expression observed in the IS + CP region in LP animals at PND 120 could therefore have been a response to the slight decreases in both Src 416 and Src 527 expression found in this epididymal region.

Several studies have shown the existence of crosstalk between Src and AR (Guo et al., 2006; Chattopadhyay et al., 2017; Szafran et al., 2017). Src is able to mediate AR phosphorylation, resulting in nuclear translocation and AR-responsive transcription (Guo et al., 2006; Chattopadhyay et al., 2017). Recently, an inverse AR and Src regulatory network has been supported in which AR may act on Src through microRNA (miR) expression modulation, thus regulating Src expression in a posttranscriptional way (Gu et al., 2014; Liu et al., 2015; Siu et al., 2016). AR transcribes and regulates miR-1 and miR-203, which in turn decrease Src expression; therefore, AR negatively regulates Src through miRs (Liu et al., 2015; Siu et al., 2016).

Due to the slight increase in AR expression in LP animals at PND 21, we expected to find decreased Src expression. However, this decrease was noticed only for Src 527, while Src 416 was significantly increased. Src activity is regulated by tyrosine phosphorylation at two different sites with opposite effects. Phosphorylation of Y416 in the activation loop of the kinase domain promotes enzyme activation, whereas phosphorylation of Y527 in the carboxy-terminal tail renders the enzyme less active (Xu et al., 1997). The increase in Src 416 expression in LP animals at PND 21 could have been an attempt to preserve epididymal development and functionality in the face of the poor nutritional conditions to which these animals were exposed during their early postnatal life. This increase in Src 416 expression could also have been related to the increased relative epididymis weight observed in these animals.

After weaning, both NP and LP animals were fed normal-protein diets. The increase in protein supply appeared to have been enough to meet the epididymal growth needs at PND 44 as well as the epididymal maintenance needs in adulthood, as we observed decreased Src 416 expression in the IS + CP region in 44-day-old LP animals and in the CO + CD region in 120-day-old LP animals. In addition, the decreased Src 416 appeared to be accompanied by a nonsignificant increase in Src 527 expression and by a decrease in AR expression in those same regions. Importantly, Src is incorporated into sperm during sperm maturation in the epididymides, being essential for sperm motility and for sperm function in fertilization (Krapf et al., 2012). Therefore, the decrease in Src 416 expression in the CO + CD regions of LP animals at PND 120 could have been related to sperm alterations previously observed by other authors using this experimental model (Toledo et al., 2011; Rodriguez-Gonzalez et al., 2014), which could compromise the fertility of adult animals whose mothers were subjected to protein restriction during gestation and lactation.

Although testosterone is the main regulating hormone of epididymal functions, estrogen is also produced and acts in males, regulating the functions of the epididymides, particularly those related to the reabsorption activity that occurs in these organs (Hess and Zhou, 2002; Cooke et al., 2017). In immature males, the main sources of estrogen are Sertoli cells, while in adults, germ cells show elevated expression of the enzyme aromatase, being the major sources of this steroid in the male genital system (Nitta et al., 1993; Hess et al., 1995; Janulis et al., 1998). Estrogen acts through ERα and ERβ nuclear receptors, both of which are present in the epididymides (Kuiper et al., 1996; Pelletier et al., 2000; Zaya et al., 2012).

Administration of estradiol to adult male rats results in increased ER expression in the genital systems of these animals (Cardone et al., 1998; Kaushik et al., 2010; Falvo et al., 2016). Similarly, antiestrogenic substances can reduce ER expression, demonstrating the ability of estrogens to regulate the expression levels of their own receptors (Zhang et al., 2013). In addition, androgenic regulation also has an impact on the expression of ERα and aromatase p450, with this type of receptor and this enzyme, respectively, being positively modulated by testosterone in organs of the male genital system (Shayu and Rao, 2006; Kaushik et al., 2012; Falvo et al., 2016). A recently published study by our research group showed that maternal protein restriction during gestation and lactation significantly decreased serum testosterone levels in 44-day-old animals (Cavariani et al., 2019).

Thus, the discrete increase in estradiol levels accompanied by the slight increase in serum testosterone levels in 21-day-old LP animals (Cavariani et al., 2019) could have been responsible not only for the increased expression of the aromatase p450 throughout the epididymides but also for the increase in ERα expression observed in the CO + CD epididymal regions of these animals.

Serum estradiol levels in the 44-day-old animals were below the detection level of the chemiluminescence technique and therefore could not be quantified. Although the lack of estradiol concentration at PND 44 impaired the effects of maternal protein restriction on these animals, the low levels were expected. In male rats, the period between PNDs 42 and 55 corresponds to the peripubertal period, and estradiol is very low, while testosterone reaches its peak (Dohler and Wuttke, 1975; Bell, 2018). However, a recent study published by our research group showed that, at this age, testosterone levels are significantly lower in animals whose mothers have received a low-protein diet than in animals whose mothers have received a normal-protein diet (Cavariani et al., 2019). The increase in ERα expression in the CO + CD region in LP animals at PND 44 could represent a compensatory mechanism given the reduced serum concentrations of testosterone. In adulthood, a protease

removes DNA from the binding portion of ERα in the epididymides, showing that the action and influence of estrogen on these organs changes with age and that its action is greater during epididymal development than during adulthood (Hendry and Danzo, 1986; Schon et al., 2009). This mechanism may explain the fact that ERα expression was increased in the CO + CD regions of LP animals at PNDs 21 and 44 but did not remain altered at PND 120.

Regarding ERβ expression, no differences were observed between NP and LP animal epididymides at PNDs 21 and 44. Estrogens have the ability to differentially regulate the expression of the two ER types (Zhang et al., 2013). Thus, the decrease in ERβ expression observed in the IS + CP epididymal region in LP animals at PND 120 could have been a direct result of the slight decreases in serum estradiol levels observed in these animals without alteration of ERα expression.

Cldn-1 is a transmembrane protein that integrates the blood–epididymal barrier, whose structure and integrity are crucial for maintenance of the specific epididymal intraluminal environment (Hoffer and Hinton, 1984; Gregory and Cyr, 2006; Cyr et al., 2007; Dufresne and Cyr, 2007). It was observed that a low-protein diet increased Cldn-1 expression in the epididymides of the animals at all analyzed ages, although this increase was significant only in the CO + CD epididymal region at PND 44. Data regarding the expression patterns of AQPs 1 and 9 in a study using this same experimental model demonstrated that despite the fact that a decrease in AQP9 expression in the IS + CP region and increases in AQP1 and AQP9 expression in the CO + CD region were significant only for LP animals at PND 44, these changes were also observed in LP animals at 21 and 120 days of age. The decrease in AQP9 expression in the IS + CP region could have resulted in reduced water absorption; consequently, a greater amount of water could have been present in the epididymal lumen of this region and could have reached the epididymal CO + CD region, which could have led to the appearance of edema in the epididymides (Cavariani et al., 2019). Therefore, the increase in Cldn-1 expression observed in the epididymides of LP animals could have been an attempt to preserve the structure and conformation of the organ until AQP9 drained the excess water that was not absorbed by the IS + CP region, which would then be removed from the epididymal intertubular space by AQP1, preventing edema appearance and keeping the intraluminal environment balanced.

Collectively, the offspring of mothers who had limited protein intake during gestation and lactation showed reduced size and low birth weight remaining until adulthood. In addition, low genital organ weights were observed in rats at all ages, thus revealing the importance of maternal diet quality and showing that changes in the diet components can permanently affect the phenotype of an adult organism. Maternal protein restriction damaged the structure and functioning of the developing epididymis, since the expression of proteins associated with regulation, development and maintenance of the organ was altered in an age-dependent manner. Although some changes did not remain until adulthood, insufficient supply of proteins in early life impaired the structure and functioning of the epididymis in important periods of postnatal development, which may have contributed to the appearance of spermatic changes related to sperm motility, viability and concentration that could compromise the fertility of adult animals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Experimentation (EAEC) of the Institute of Biosciences of Botucatu (number 797-CEUA).

AUTHOR CONTRIBUTIONS

MC, TDM, and RD contributed actively to the elaboration of the study main idea, participated to the animal care, collected, analysis and interpretation of the data. MC wrote the manuscript. LG, PF, and WS contributes to the acquisition of material, with the experimental design and analysis and interpretation of the data. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Maternal Diabetes and Postnatal High-Fat Diet on Pregnant Offspring

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Maternal diabetes-induced fetal programming predisposes offspring to type 2 diabetes, cardiovascular disease, and obesity in adulthood. However, lifelong health and disease trajectories depend on several factors and nutrition is one of the main ones. We intend to understand the role of maternal diabetes-induced fetal programming and its association with a high-fat diet during lifelong in the female F1 generation focusing on reproductive outcomes and the possible changes in physiological systems during pregnancy as well as the repercussions on the F2 generation at birth. For this, we composed four groups: F1 female pups from control (OC) or from diabetic dams (OD) and fed with standard (SD) or high-fat diet from weaning to full-term pregnancy. During pregnancy, glucose intolerance and insulin sensitivity were evaluated. In a full-term pregnancy, the maternal blood and liver were collected to evaluate redox status markers. The maternal blood, placental tissue, and fetal blood (pool) were collected to evaluate adiponectin and leptin levels. Maternal reproductive parameters were evaluated as well. Maternal diabetes and high-fat diet consumption, in isolation, were both responsible for increased infertility rates and fasting glucose levels in the F1 generation and fetal growth restriction in the F2 generation. The association of both conditions showed, in addition to those, increased lipoperoxidation in maternal erythrocytes, regardless of the increased endogenous antioxidant enzyme activities, glucose intolerance, decreased number of implantation sites and live fetuses, decreased litter, fetal and placental weight, increased preimplantation losses, and increased fetal leptin serum levels. Thus, our findings show that fetal programming caused by maternal diabetes or lifelong high-fat diet consumption leads to similar repercussions in pregnant rats. In addition, the association of both conditions was responsible for glucose intolerance and oxidative stress in the first generation and increased fetal leptin levels in the second generation. Thus, our findings show both the F1 and F2 generations harmed health after maternal hyperglycemic intrauterine environment and exposure to a high-fat diet from weaning until the end of pregnancy.

Keywords: Female reproduction, diabetes, malnutrition, fetal programming, rodent

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1 INTRODUCTION

Diabetes is a complex and chronic disease that requires continuous medical care, with strategies of multifactorial risk reduction and glycemic control (ADA, 2021a). According to the American Diabetes Association (ADA), there are three main classes of *Diabetes mellitus* (DM): type 1 DM (DM1), which is characterized by the autoimmune destruction of pancreatic beta (β)-cells, usually causing insulin deficiency; type 2 DM (DM2) that is characterized by the progressive loss of insulin secretion by β-cells, leading to insulin resistance as a background; and gestational *diabetes mellitus* (GDM) that is diagnosed in the second or third trimester of pregnancy, in which the diabetes is not manifested before pregnancy (ADA, 2021b). The prevalence of diabetes in pregnancy has increased worldwide. In 2019, the International Diabetes Federation (IDF) showed that one in six pregnancies was being affected by hyperglycemia, with 13.6% of pregnancies affected by pre-gestational diabetes and 86.4% affected by GDM. The occurrence of GDM has been described by the International Diabetes Federation as a severe and neglected threat to maternal and child health (IDF, 2019). The hyperglycemic intrauterine environment can interact with the genome of offspring, inducing long-term altered patterns of gene expression and can be transmitted transgenerationally (Gauguier et al., 1990; Boloker et al., 2002). For this reason, the IDF recommends that pregnant women with diabetes or at high risk of developing GDM should monitor their blood glucose to avoid long-term consequences for themselves and transgenerational effects for their children (IDF, 2019).

In addition to maternal glycemic control during pregnancy, adequate nutrition and specific nutrients are important in all periods of life, but they are essential during specific times, such as in intrauterine life and early postnatal life (Greco et al., 2019). Studies have shown that the first 1,000 days after conception (from intrauterine life/pregnancy to the first 2 years of the life of a child) are windows of particular sensibility to environmental factors influencing lifelong trajectories through health and disease (Barker, 2012; Simeoni et al., 2018). In this context, several epidemiologic and experimental studies show that maternal hyperglycemia and an unbalanced diet can induce health consequences several decades after exposure, leading to a higher prevalence of overweight, obesity, DM2, GDM, and reproductive disorders in the adult lives of male and female descendants (Boullu-Ciocca et al., 2008; Glavas et al., 2010; Bouchard et al., 2012; Kayser et al., 2015; Sánches-Guarrido et al., 2015; World Health Organization, 2019). However, the exposure to adverse conditions during intrauterine development is particularly important to female offspring because it can cause physiological changes that have the potential to alter both the reproductive capacity of the first generation and the health of the second generation through changes in the oocyte (Yao et al., 2021). Moreover, the sex of the embryo also plays an important role in determining how an insult might become part of the epigenome and be transmitted to future generations. In the case of female rats, as the entire repertoire of primordial follicles forms during intrauterine and early neonatal phases, the consequences may be more evident in reproductive aspects than in males (Yao et al., 2021).

Environmental factors and lifestyle have a direct influence on developmental programming and may even reverse those (Gluckman et al., 2008). Leptin and adiponectin are hormones that regulate energy balance and insulin sensitivity, playing a critical role in the establishment of this program (Parent et al., 2014; Choi, 2018). Both hormones have a key role in metabolism, maternal–fetal interaction, and metabolic abnormalities which can lead to pregnancy complications and fetal growth changes (Santos et al., 2015). Intrauterine exposure to hyperglycemia leads to the development of both leptin and insulin resistance in the placenta (Greco et al., 2019), and these alterations are related to maternal glycemic levels (Bouchard et al., 2012; Houde et al., 2013). Moreover, animal and human studies show that leptin and insulin resistance act on hypothalamic receptors and appetite circuits, leading to postnatal hyperphagia, decreased satiety, and subsequent development of metabolic syndrome (Block and El-Osta, 2017). Plasma leptin concentrations, in both dams and their offspring, may play a role in linking nutrition and development (Stocker et al., 2007). Stocker et al. (2007) verified that the impaired glucose tolerance in rats from mothers fed a high-fat diet can be prevented by the administration of leptin to their mothers, indicating the maternal leptin levels during pregnancy and lactation can affect the development of energy balance regulatory systems in their offspring.

In an attempt to assess the repercussions of fetal programming and malnutrition and to eliminate the possible confounding factors between these two simultaneous interventions during pregnancy, we first used a pre-gestational diabetes model (F0 generation), and then a high-fat diet (HFD) was offered only to the female offspring (F1 generation) from weaning until full-term pregnancy. Thus, we intend to understand the role of maternal diabetes-induced fetal programming and its association with a high-fat diet during lifelong in the female F1 generation focusing on reproductive outcomes and the possible changes in physiological systems represented by glycemic metabolism, redox status, concentrations of leptin and adiponectin, and insulin sensitivity as well as the repercussions on the F2 generation at birth.

2 METHODS

2.1 Ethics

The Ethics Committee for the Use of Animals of Botucatu Medical School approved all the methods adopted in this study (Protocol CEUA Number: 1218/2017). Animal handling was performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals promulgated by the Society for the Study of Reproduction and with the guidelines provided by the Brazilian College of Animal Experimentation.

2.2 Calculation of Sample Size

Based on previous experiments conducted in our laboratory, each rat from a different litter was used for circulating glycemic calculation obtained by area under the curve (AUC) during

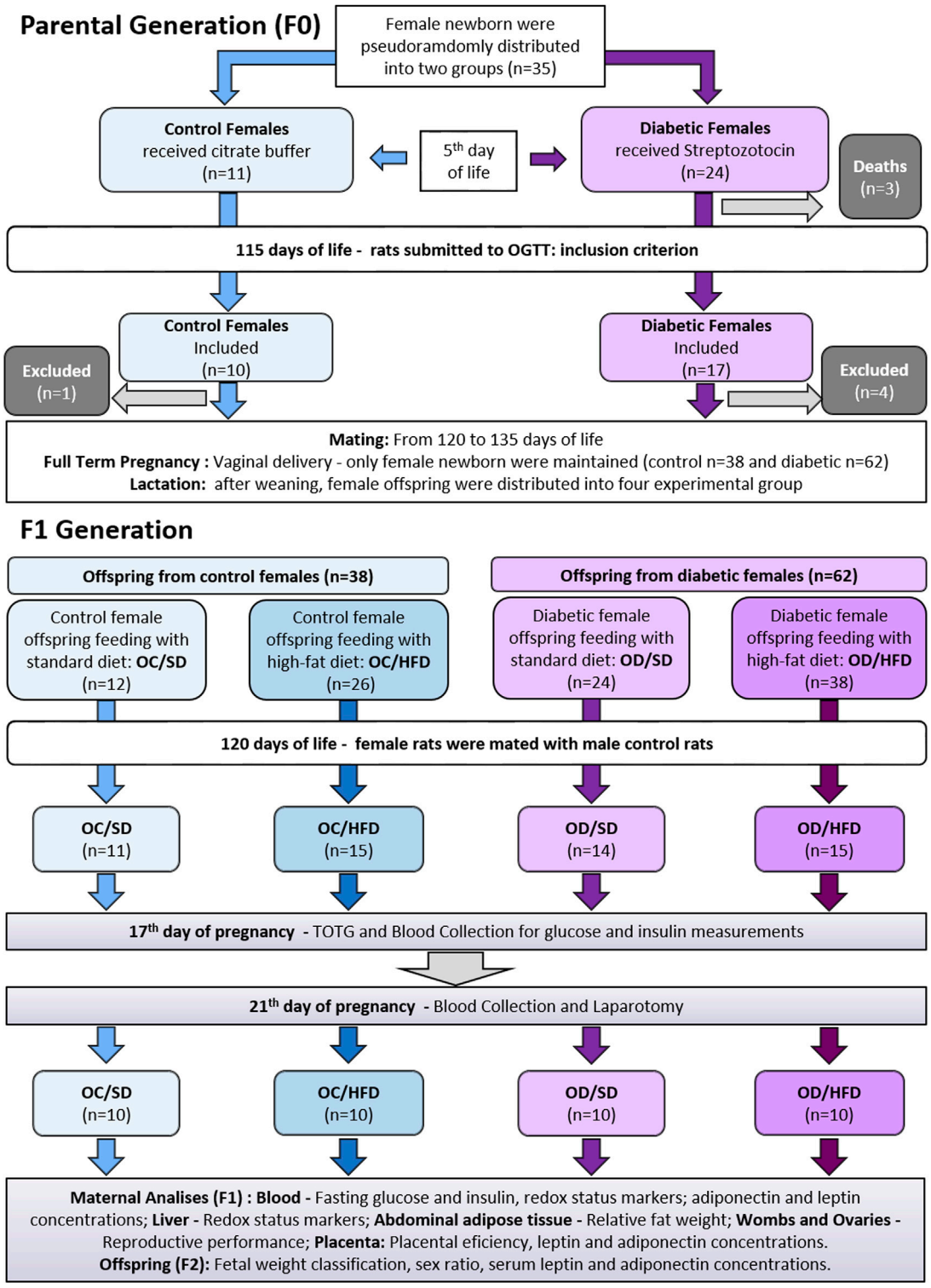

FIGURE 1 | Experimental approach for parental and offspring generation.

the oral glucose tolerance test (OGTT), and using 90% power and an error type I of 5%, the effect size was determined as 10 rats per group.

2.3 Animals

The animals were maintained at the local laboratory of Botucatu Medical School (Unesp) under controlled conditions of

temperature (22 ± 2°C), humidity (50 ± 10%), and light/dark cycle (12 h) in polypropylene cages lined with wood shavings. Filtered water and feed were offered *ad libitum*. As a form of environmental enrichment, paper balls were used in the cages (Simpson and Kelly, 2011).

2.4 Experimental Approach

2.4.1 Experimental Approach for Parental Generation (F0)

The parental generation (F0) aimed to create an inadequate intrauterine environment leading to fetal programming based on the induction of experimental diabetes. The experimental sequence was described in **Figure 1**.

Male and female Sprague-Dawley rats (200–250 g) were mated overnight to obtain female pups to induce or not induce diabetes during the perinatal period of these offspring. The following morning, when spermatozoa were found in the vaginal smear, was designated as day 0 of pregnancy. The female rats that were not mated after 15 consecutive days were considered infertile and excluded from this study (Moraes-Souza et al., 2017). The pregnant rats were randomly assigned to diabetic (D) and nondiabetic groups by draw, and the female rats were kept in individual polypropylene cages during pregnancy. On day 21 of pregnancy, the rats had a vaginal delivery. Eight pups per dam were kept with their dams until postnatal day (PND) 22, considering the highest number of females wherever possible. We prioritize female rats because our research group studies the transgenerational effects related to pregnancy. The excess pups were euthanized by decapitation (Sinzato et al., 2021).

2.4.2 Diabetes Induction

On postnatal day 5 (PND), the female pups (First Generation—F1) were injected with streptozotocin (Sigma Aldrich®, United States, a dose of 70 mg/kg, intraperitoneal route) for diabetes induction, while control female rats received an equivalent volume of vehicle (citrate buffer - 0.01 M pH 4.5), as previously described (Damasceno et al., 2011). Male offspring were maintained with their mothers until weaning and then were euthanized by decapitation or used by other investigators. The blood glucose levels were determined in female adult rats on a PND 75, as established by Gallego et al. (2018—with modifications) and classified by the American Diabetes Association (ADA, 2019). Following, female rats were considered diabetic when presenting glycemia ≥200 mg/dl (11.11 mmol/L) at least at a one-time point during the oral glucose tolerance test (OGTT). In the control group, only rats with glycemia <140 mg/dl (7.77 mmol/L) at least at a three-time point during the OGTT were included. The female rats that were not accomplishing the aforementioned characteristics were anesthetized by using sodium thiopental (Thiopentax®, Cristália, Brazil—120 mg/kg dose), euthanized, and excluded from this study.

2.4.3 Mating, Pregnancy, and Lactation

After inclusion criteria on PND 90, the diabetic (D) and nondiabetic (control—C) female rats were mated as previously described. The offspring were obtained through vaginal delivery. To avoid differences in maternal care between male and female pups (Beery and Francis, 2011) which could result in epigenetic consequences (Champagne et al., 2003), after birth, all-male newborns were euthanized by decapitation, while female newborns were kept in a range from six to eight pups per litter until weaning day 22, to maintain the milk consumption balance. The excess number of female pups was also euthanized by decapitation. When the litter had less than six female pups, it was excluded from the study and redirect to another experiment in our group. After weaning, female pups from nondiabetic (control) and diabetic dams were randomly assigned to compose the experimental groups.

Experimental approach for female pups from the maternal diabetic environment (F1 generation).

2.4.4 Experimental Groups and Dietary Patterns

Upon weaning, the female pups from diabetic (OD) and control (OC) mothers were pseudo-randomized by lot respecting a maximum of four female pups per mother (with two sisters to each group: C or D). The other females from the same litter were used in another subproject with non-pregnant rats (Protocol CEUA Number: 1218/2017). The OD and OC groups were further distributed into two other groups according to their diets: standard diet (SD - Kcal content: 28.54% protein, 62.65% carbohydrate, 8.7% fat, Purina®, Brazil) or high-fat diet (HFD - Kcal content: 23.43% protein, 46.63% carbohydrate, 30% fat, using lard as the main fat source). Additional bromatological comparison between the diets is shown in **Supplementary Table S1**. Thus, four experimental groups were established: OC/SD: female pups from control mothers and fed standard diet; OC/HFD: female pups from control mothers and fed high-fat diet; OD/SD: female pups from diabetic mothers and fed standard diet; and OD/HFD: female pups from diabetic mothers and fed high-fat diet. All the groups were followed from weaning to PND 120. The HFD was handmade at our institution, adequately supplemented with vitamins and minerals, and maintained under refrigeration until the time of use (Paula et al., 2021). Given the fact that the diets had visual characteristics that easily allow their distinction, random housing, and blinding of caregivers and/or investigators were not possible.

2.4.5 Mating

From PND 120, diabetic (D) and control (C) female rats were mated as previously described.

2.4.6 Pregnancy

2.4.6.1 Oral Glucose Tolerance Test (OGTT) and QUICK Index.

Maternal weight, water consumption, and food intake (evaluated as gram intake and energy intake) were monitored during the entire pregnancy. Energy intake was determined to observe if the moderate differences showed in body weight evolution would be related to differences in food intake (Pérez-Matute et al., 2007). On pregnancy day (PD) 17, OGTT was performed for glucose tolerance evaluation as previously described (Tai, 1994; Gallego et al., 2019). In this same test, blood samples were collected at fasting for insulin measurements using an ELISA commercial kit

(Crystal Chemical® Code: 90060, United States). The quantitative insulin sensitivity check index (QUICK index) was calculated as defined by Katz et al. (2000) as QUICKI = 1/[(log (I_0) + log (G_0)], where I_0 is the fasting plasma insulin level (microunits/mL), and G_0 is the fasting blood glucose level (milligrams per dL).

2.4.6.2 Blood Collection and Laparotomy

On PD 21, the rats were intraperitoneally anesthetized by using sodium thiopental (Thiopentax®, Cristália, Brazil—120 mg/kg dose) and decapitated. The collection of maternal blood was performed to obtain serum and washed erythrocyte samples according to the ELISA kit's instructions for leptin and adiponectin (#80570; # E-EL-R0582) assays and according to Souza et al. (2010) for redox status analyses, respectively. All samples were stored at −80°C until further analysis. Uterine horns, ovaries, newborns, and placentas were removed and weighed for evaluation of maternal reproductive performance, birth weight classification, and placental efficiency (Sinzato et al., 2021). Visceral fat pads from the abdominal cavity (periovarian, periuterine, perirenal, and retroperitoneal) were pooled and weighed to calculate the relative fat weight (grams of total visceral fat/100 g of body weight). Maternal liver and two placentas from each litter (one from female newborns and the other from one of the male newborns were randomly assigned) were removed and quickly frozen in liquid nitrogen and stored in a freezer at -80°C until homogenates were performed. The liver homogenate (modified from Spada et al., 2014) was used for redox status evaluation, and the placental homogenate (modified from Portela et al., 2021) was used for leptin and adiponectin determinations. Newborns were sexed and all male and female newborns from each litter were euthanized and killed by decapitation for blood collection (pool). Serum samples from each pool were obtained as described for maternal blood and used for leptin and adiponectin determinations.

2.4.6.3 Reproductive Outcomes and Birth Weight Classification

The mothers who presented no live newborns at the end of pregnancy were not included in the reproductive analyses. The ovaries were used to count the number of corpora lutea as an indirect parameter to assess the number of oocytes. The uterus of pregnant rats was dissected to count the number of living and dead fetuses, resorptions (embryonic deaths), and implantation sites (Sinzato et al., 2021). When the implantation sites were visually undetectable, the Salewski (1964) reagent was used as a dye. The percentage of embryonic loss before implantation (preimplantation loss rate), the percentage of embryonic loss after implantation (postimplantation loss rate), and the classification of the birth weight into small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA) were made according to Sinzato et al. (2021).

2.4.6.4 Liver Homogenates for Redox Status Analyses

Liver samples from a maternal organism (F1 generation) frozen samples were homogenized using the bullet blender system, adding zirconium beads, phosphate-buffered saline (PBS—0.1 M Na2HPO4, 0.1 M KH2PO4, 0.1 M EDTA, pH 7.8), and protease inhibitor cocktail (#P8340) for glutathione peroxidase (GSH-Px), catalase (CAT), reduced thiol groups (-SH), and hydrogen peroxide (H_2O_2—reactive oxygen species) assays. For thiobarbituric acid reactive substances (TBARS–lipoperoxidation biomarker) assay, RIPA lysis buffer (1X #92590) was used. For superoxide dismutase (SOD), a solution with 20 mM HEPES, 1 mM EGTA, 210 mM mannitol, and 70 mM sacarose (pH 7.2) was used. Then, all samples were centrifuged at 1,600 *x g* for 10 min at 4°C for GSH-Px, CAT, -SH, and H_2O_2; 240 *x g* for 10 min at 4°C for TBARS; and 1.500 *x g* for 5 min at 4°C for SOD. After centrifugation, the supernatant was stored in a freezer at -80°C until assays. Protein concentrations were analyzed by the Bradford method (Bradford, 1976).

2.4.6.5 Placental Homogenate for Leptin and Adiponectin Analyses

From here, all procedures were performed with the investigator blinded to the analyses. For the placental homogenate, out of ten samples collected from male newborns, five were randomly assigned to analyses. The same procedure has been made for the placentas from female newborns. Placental frozen samples were homogenized using the bullet blender homogenizer® (Next Advanced, NY, United States) adding zirconium oxide beads (1 mm–Code ZrOB10, and 2 mm–ZrOB20, Next Advanced, NY, United States) and lysis buffer (RIPA 1X #9806), protease/phosphatase inhibitor cocktail (1X #5872), phenylmethane sulfonyl fluoride (PMSF 1 mM #8553), and the remaining volume completed with water purified by the ultra purifying master system® (GEHAKA, São Paulo, Brazil). After homogenization, the samples were kept and incubated on ice and homogenized by vortex every 15 min for 2 hours, then centrifuged at 7,000 × g for 15 min at 4°C, and the supernatant was stored at -20°C. Protein concentration was analyzed by the Bradford method using the BSA curve as a standard. The final protein concentration in μg/μL was determined based on the BSA standard curve (Bradford, 1976) performed at each dose. Samples were normalized by the lowest protein concentration obtained after measurement.

2.4.6.6 Evaluation of redox status and hormone assays

Samples of washed red blood cells and liver homogenates were used to evaluate the redox status of a maternal organism following the protocols from Yagi (1976) and Buege and Aust (1978) for TBARS; Boyne and Ellman (1972) modified by Hu (1994) for -SH; Noble and Gibson (1970) for H_2O_2; Marklund and Marklund (1974) for SOD; Lawrence and Burke (1976) for GSH-Px; and Aebi (1984) for CAT.

Fasting serum insulin was determined using an ultra-sensitivity ELISA kit from Crystal Chemicals→, United States (Code: 90060). For maternal, placental, and fetal adiponectin and leptin determinations were used, respectively, an ELISA kit from Crystal Chemicals®, United States (Code: 80570) and an ELISA kit from Elabscience®, United States (Code: E-EL-R0582).

TABLE 1 | Maternal reproductive performance, relative fat weight, placental and birth weight, and placental efficiency on the 21st day of pregnancy from F1 control pregnant offspring (OC) and diabetic pregnant offspring (OD) that received standard diet (SD) or high-fat diet (HFD) from weaning.

Variable	Group			
	OC/SD	OC/HFD	OD/SD	OD/HFD
Fertility rate (%)	91.7	58.3	57.7	39.5*
Non-pregnant at full-term (%)	9.1	33.3	26.7	33.3
Number of corpora lutea	13.9 ± 1.7	14.0 ± 1.6	13.8 ± 0.9	13.2 ± 1.7
Number of implantation	13.7 ± 1.8	12.9 ± 1.5	11.1 ± 4.5	7.1 ± 5.3*#
Number of embryonic deaths	1.0 ± 1.2	1.9 ± 2.0	1.4 ± 1.9	0.8 ± 1.3
Number of live fetuses	12.7 ± 1.6	10.9 ± 2.4	9.7 ± 4.9	6.6 ± 5.2*#
Preimplantation loss (%)	1.1	7.5	12.5	28.7*
Postimplantation loss (%)	7.0	15.3	18.2	12.1
Maternal weight gain (g)	126.3 ± 31.3	82.1 ± 24.5*	109.3 ± 17.7	88.0 ± 16.5*$
Litter weight (g)	99.6 ± 12.8	79.0 ± 16.3	79.5 ± 25.9	58.9 ± 29.8*
Relative fat weight (g/100 g of body weight)	2.49 ± 0.58	4.14 ± 1.02*	3.68 ± 1.43	3.18 ± 0.23
Birth weight (g)	5.84 ± 0.33	5.14 ± 0.66*	5.29 ± 0.68*	4.81 ± 0.87*#$
Placental weight (g)	0.56 ± 0.10	0.53 ± 0.08	0.60 ± 0.08*	0.51 ± 0.12$
Placental efficiency	10.74 ± 1.43	9.89 ± 1.57*	8.86 ± 1.13*	9.68 ± 2.10*$

Values expressed as mean ± standard deviation (SD). n = *10 rats/group.* *p < *0.05—compared to the OC/SD group;* #p < *0.05—compared to the OC/HFD group;* $p < *0.05—compared to the OD/SD group (Tukey Multiple Comparison Test, and Poisson distribution test was used for corpora lutea, implantation and alive fetus numbers, and placental efficiency and chi-square test for proportions).*

2.5 Statistical Analysis

To calculate the sample size, 10 mothers of the parental generation were used, each one from a different litter, and a completely randomized design was made by the Research Support Office of Botucatu Medical School, Unesp. Approximately 10 animals/groups have been established for each group. For the analysis of the F1 generation, we used a maximum of two female rats from the same litter for the experiments. All newborns from each litter were used in the F2 generation analyses. There were no used repeated measures for any parameter. For asymmetric distribution of the data (fetal and placental weights, glycemia of OGTT and AUC, serum insulin concentrations during OGTT, and TBARS levels), the gamma distribution test followed by the Wald multiple comparison test was used. For the QUICK Index, adiponectin and leptin concentrations, other redox status markers, maternal weight gain, maternal body and litter weight, water intake, food consumption, and energy intake, one-way ANOVA followed by the Tukey multiple comparison test was used. The Poisson distribution test was used for corpora lutea, implantation and alive fetus numbers, and placental efficiency since these parameters presented the asymmetric distribution of the data. For proportion analysis (sex ratio, fetal weight classification, and pre and postimplantation loss percentages), the chi-square test was used. A minimum confidence limit of 95% ($p < 0.05$) was considered statistically significant for all statistical comparisons. All statistical analyses were performed using SAS software for Windows, v.9.4.

3 RESULTS

3.1 Fertility Rate, Maternal Weight and Water, and Food Consumption

The fertility rates were 91.7%, 57.7%, 58.3%, and 39.5% in OC/SD, OC/HFD, OD/SD, and OD/HFD, respectively. The OC/HFD and OD/SD groups presented no differences compared to the control group (OC/SD) ($p = 0.060$ and $p = 0.059$, respectively). The OD/HFD group presented a lower fertility rate compared to the OC/SD ($p < 0.05$) (**Table 1**). The percentage of female rats with positive vaginal smear did not reach full-term pregnancy is presented in **Table 1** ($p > 0.05$). These animals were not included in the statistical analysis of the preimplantation losses.

The maternal weight presented no differences between OC/HFD and OC/SD at PD0, PD_7, PD_{14}, and PD_{21} compared to other groups. The OD/HFD rats showed an increase in relative fat weight at full-term pregnancy (**Table 1**) and maternal weight PD_7 of pregnancy compared to the OC/SD, OC/HFD, and OD/HFD dams (**Supplementary Figure S2A**). There were no differences in water consumption among groups (**Supplementary Figure S2D**). The food consumption was lower in OC/HFD rats at PD_{14} and PD_{21} of pregnancy compared to the OC/SD. The OD/HFD group presented lower food intake on day 14 of pregnancy than the OC/SD rats and compared to the OD/SD group at day 21 of pregnancy (**Supplementary Figure S2B**). However, when we evaluated the energy intake, the total calories ingested (Kcal/day) in the OC/HFD and OD/HFD groups (fed with a high-fat diet) at PD0 and PD_7 had an increase compared to those in the OC/SD and OD/SD groups. At PD_{14}, only the OD/HFD rats presented a higher energy intake on the same day of pregnancy in relation to the OD/SD (**Supplementary Figure S2C**).

3.2 TOTG, AUC, Fasting Insulin, and QUICK Index

Figure 2 shows the comparison of the glycemic levels by OGTT (**Figure 2A**) and AUC (**Figure 2B**), QUICK Index (**Figure 2C**), and fasting insulin (**Figure 2D**) among groups. The OD/SD and OD/HFD groups showed increased fasting glucose levels compared to the OC/SD group. At time point 30 of TOTG, all groups had an increase in the blood glucose levels compared to time point 0, but only the OD/SD and OD/HFD groups presented

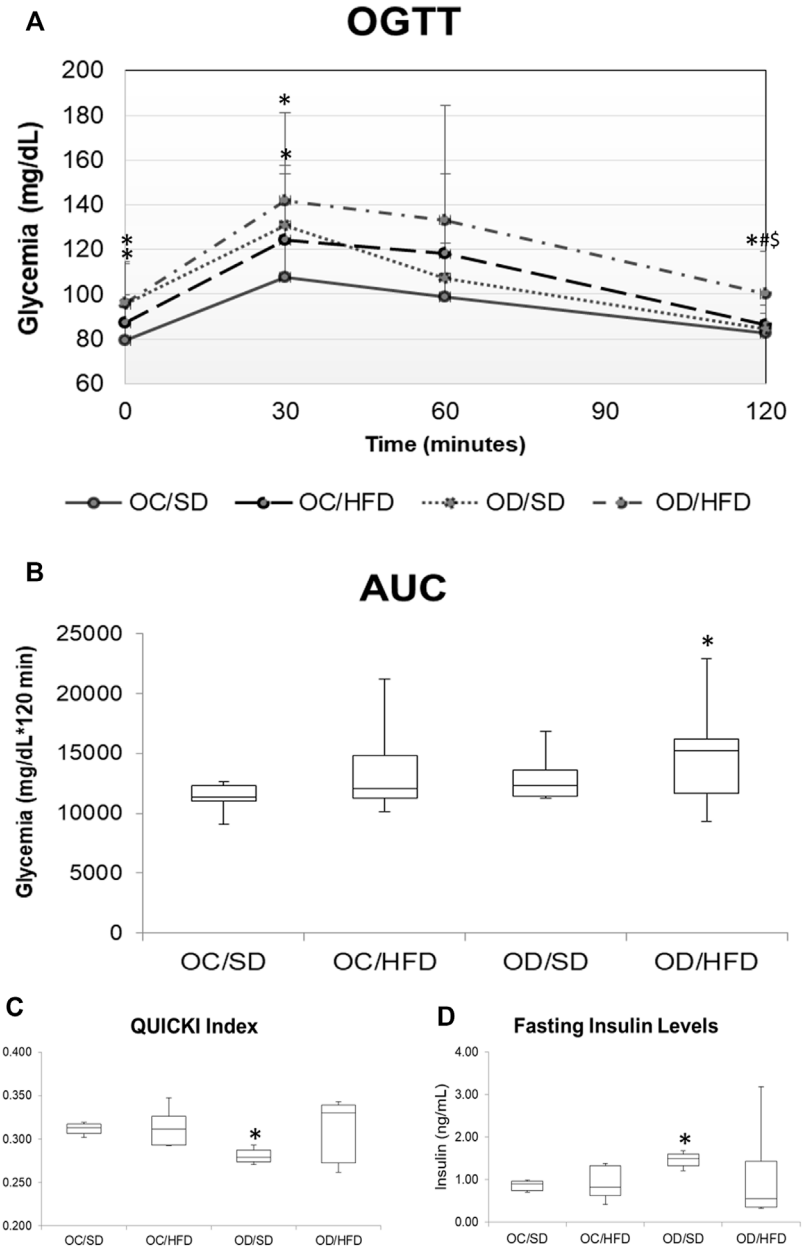

FIGURE 2 | (A) OGTT, oral glucose tolerance test; **(B)** AUC, area under the curve; **(C)** QUICK Index; and **(D)** fasting maternal insulin levels on PD17 from OC, control offspring; and OD, diabetic offspring that received a SD, standard diet; or HFD, high-fat diet from weaning. Values are expressed as mean ± SD, standard deviation. $n = 10$ rats/group. *$p < 0.05$—compared to the OC/SD group; #$p < 0.05$—compared to the OC/HFD group; \$$p < 0.05$—compared to the OD/SD group. For OGTT and AUC, the gamma distribution test was used and for QUICK Index, Tukey Multiple Comparison Test was used.

glycemic levels superior to 140 mg/dl. The OC/HFD and OD/HFD groups had increased glycemia compared to the OC/SD and OD/SD rats at 60 min of TOTG. At 120 min of TOTG, the OD/HFD group showed an increase in blood glucose levels compared to the other groups (OC/SD, OC/HFD, and OD/SD). AUC showed increased total glucose during TOTG of OC/HFD, OD/SD, and OD/HFD compared to the OC/SD group. The fasting maternal insulin concentration was increased in the OD/SD and OD/HFD rats compared to the OC/SD group, and the QUICK Index (insulin sensitivity index) was decreased in the OD/SD group compared to the other groups.

3.3 Analysis of Redox Status Markers in Maternal Blood and Liver Samples

Figures 3, **4** show, respectively, redox status markers sampled from maternal washed erythrocytes and liver. There was no difference in SOD activity, H_2O_2, and -SH concentrations among groups in

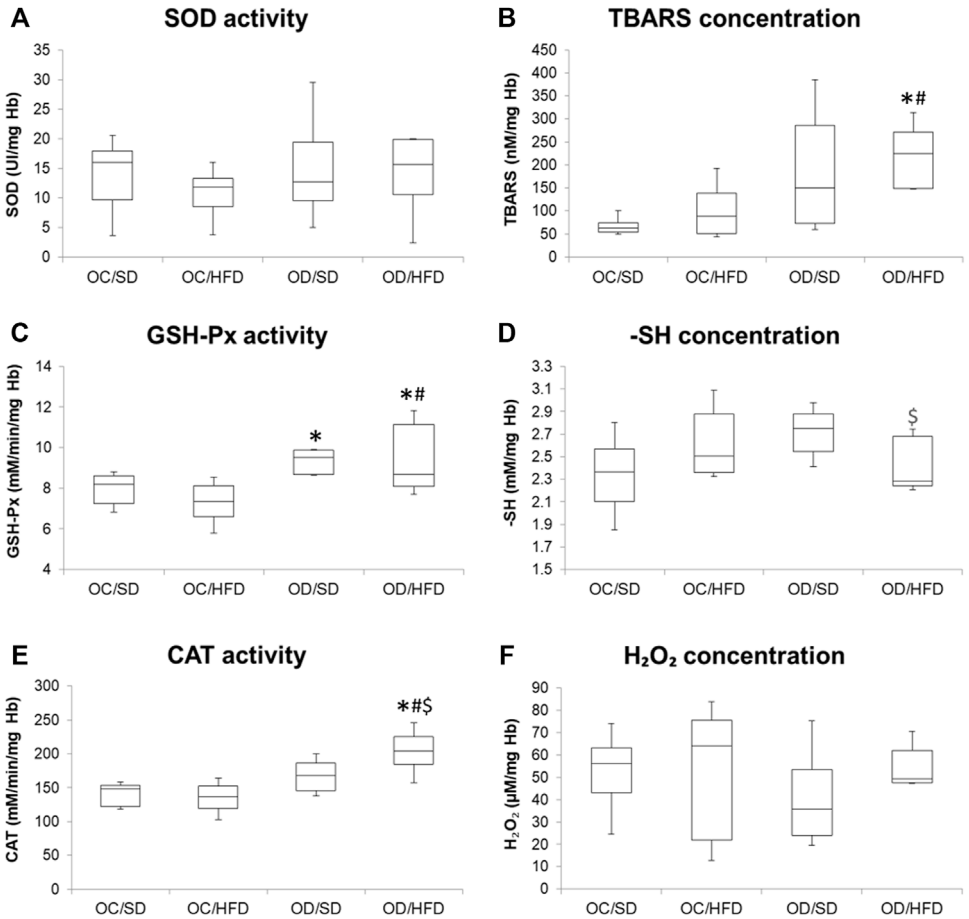

FIGURE 3 | (A) SOD, superoxide dismutase activity; **(B)** TBARS, tiobarbituric acid reagent species concentration; **(C)** GSH-Px, glutathione peroxidase activity; **(D)** reduced thiol groups concentration (-SH) **(E)** CAT, catalase activity; and **(F)** hydrogen peroxide concentration (H_2O_2) in washed erythrocytes at full-term pregnancy from OC, control offspring; and OD, diabetic offspring that received a SD, standard diet or HFD, high-fat diet; from weaning. Values are expressed as mean ± SD, standard deviation; $n = 10$ rats/group. *$p < 0.05$—compared to the OC/SD group; #$p < 0.05$—compared to the OC/HFD group; \$$p < 0.05$—compared to the OD/SD group (Tukey Multiple Comparison Test).

maternal washed erythrocytes. There was an increase in TBARS concentration in OD/HFD dams compared to the OC/SD and OC/HFD dams. GSH-Px activities were increased in OD/SD dams compared to control dams and in OD/HFD dams compared to OC/SD and OC/HFD dams. CAT activity was increased in the OD/HFD group compared to the other groups.

Considering maternal liver samples, there were no differences in H_2O_2 concentrations, GSH-Px, and CAT activities among groups. MDA concentration was decreased in OD/HFD compared to OC/HFD rats. There was an increased SOD activity in OC/HFD dams compared to OC/SD dams and a decreased SOD activity in the OD/HFD rats compared to OC/HFD rats. Increased -SH levels were observed in the OC/HFD and OD/HFD rats when compared to the control group.

3.4 Adiponectin and Leptin Concentrations From Maternal and Fetal Serum and Placental Homogenate

Figure 5 shows, respectively, leptin and adiponectin concentrations in maternal (A and B) and fetal (E and F) serum and placental homogenates (C and D) of rats at term pregnancy. As there was no difference between male and female rats, we chose to present the data together. The adiponectin levels showed no differences in the different tissues among the groups. The leptin concentrations of the maternal serum and placental homogenate presented no difference among groups. There was an increase in serum leptin concentration in the fetuses from OD/SD and OD/HFD dams compared to the OC/SD rats.

3.5 Maternal Reproductive Performance, Sex Ratio, and Fetal Weight Classification

Table 1 shows the fertility rate, percentage of non-pregnant rats, reproductive performance, relative fat weight, placental and fetal weight, and placental efficiency of the rats. There were no significant differences in the number of corpora lutea, postimplantation loss percentage, and maternal weight gain among groups. The OC/HFD and OD/SD dams showed reduced litter and fetal weight compared to the OC/SD group. The relative fat weight was increased in the OC/HFD

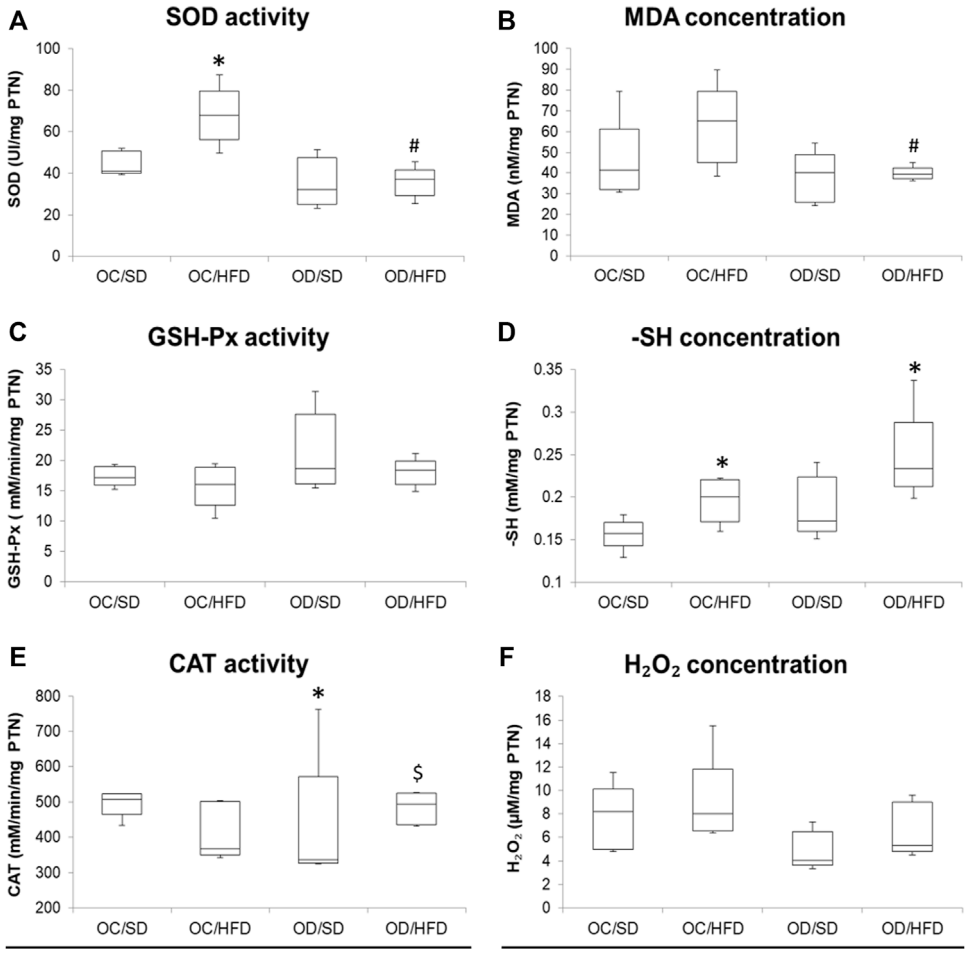

FIGURE 4 | (A) SOD, superoxide dismutase activity; **(B)** MDA, malondialdehyde concentration; **(C)** GSH-Px, glutathione peroxidase activity; **(D)** -SH, reduced thiol groups concentration; **(E)** CAT, catalase activity, and **(F)** H_2O_2, hydrogen peroxide concentration in maternal liver at full-term pregnancy from OC, control offspring; and OD, diabetic offspring that received a SD, standard diet or HFD, high-fat diet; from weaning. Values are expressed as mean ± SD, standard deviation; $n = 10$ rats/group. *$p < 0.05$—compared to the OC/SD group; #$p < 0.05$—compared to the OC/HFD group; \$$p < 0.05$—compared to the OD/SD group (Tukey Multiple Comparison Test).

rats than that in the OC/SD dams. The OD/SD group showed lower fetal weight and placental efficiency, and a greater placental weight compared to OC/SD rats. The placental weight and percentage of preimplantation losses were also decreased in OC/HFD and OD/SD in relation to OC/SD. The OD/HFD presented a decrease in the number of implantations, live fetuses, litter, and weight, and increased preimplantation loss percentage compared to the OC/SD, OC/HFD, and OD/SD groups. In addition, the OD/HFD rats showed lower placental weight compared to the OC/SD and OD/SD (**Table 1**).

The fetal weight classification showed an increased number of fetuses classified as small for gestational age (SGA) and a decreased number of fetuses classified as adequate for gestational age (AGA) in all groups when compared to the OC/SD (**Figure 6**). When we compared the fetal weight classification by sex, we observed an increased ratio of SGA and a decreased ratio of AGA in the female pups from OC/HFD and OD/HFD when compared to male pups from the same group. There was no difference in the male and female ratio between the groups and among groups (**Figure 6**).

4 DISCUSSION

Clinical and experimental studies have been performed to evaluate diabetes-induced repercussions (Metzger et al., 2009; Tam et al., 2017; Lowe et al., 2019; Bueno et al., 2020) and/or malnutrition on descendants (Zhang et al., 2008; Mdaki et al., 2016; Tellechea et al., 2017; Hsu & Tain, 2019; Castro-Rodríguez et al., 2020). However, studies addressing the influence of postnatal malnutrition on female offspring that were programmed by intrauterine hyperglycemia are still lacking. Therefore, this study used a rat model with maternal diabetes to reproduce the

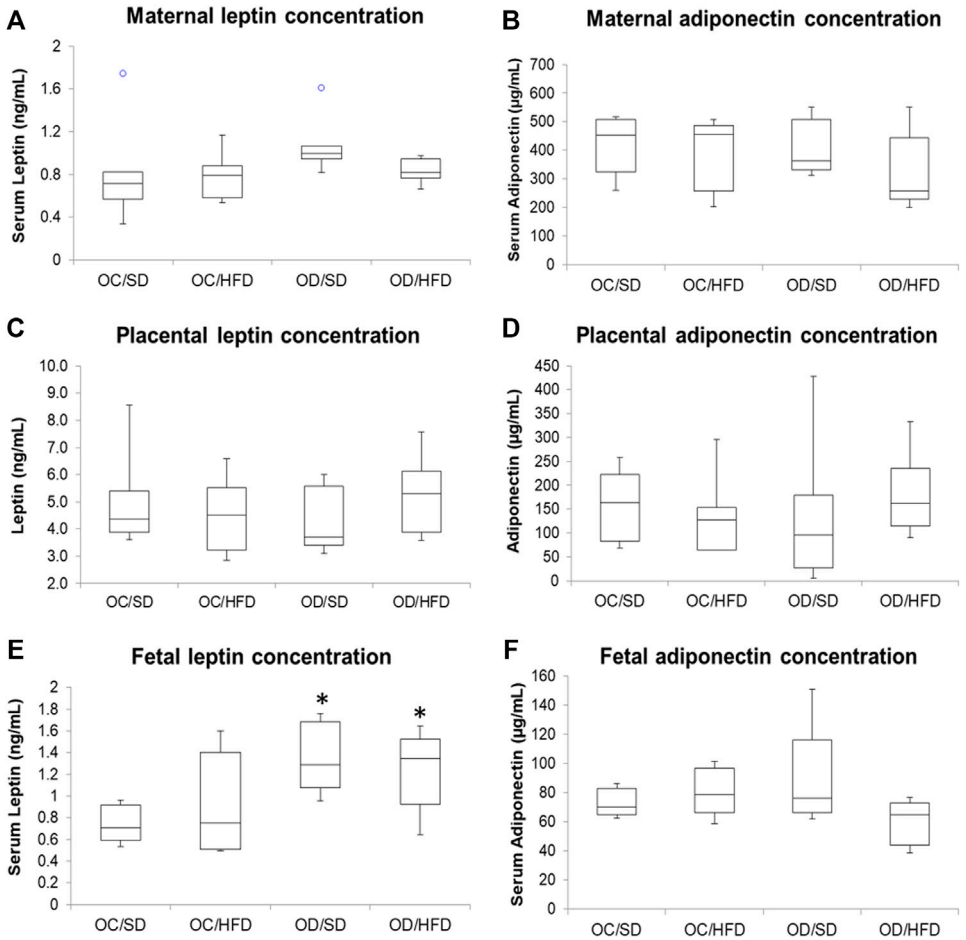

FIGURE 5 | (A) Maternal serum leptin; **(B)** maternal serum adiponectin; **(C)** placental leptin; **(D)** placental adiponectin; **(E)** fetal serum leptin; and **(F)** fetal serum adiponectin concentrations from control offspring (OC) and diabetic offspring (OD) that received a standard diet (SD) or high-fat diet (HFD) from weaning. Values are expressed as mean ± standard deviation (SD). n = 10 rats/group. *$p < 0.05$—compared to the OC/SD group (Tukey Multiple Comparison Test).° Purple dots represent the outliers of the respective groups.

transgenerational effects of hyperglycemia on female offspring (F1 generation) fed with a high-fat diet from weaning to full-term pregnancy (~ PND 150). We found that maternal-diabetes fetal programming associated with high-fat diet consumption, was responsible for increased infertility rates, increased lipoperoxidation in maternal erythrocytes, glucose intolerance, decreased number of implantation sites and live fetuses, decreased litter, fetal and placental weight and increased preimplantation losses in the F1 generation, and increased fetal leptin serum levels and fetal growth restriction in the F2 generation.

The increased infertility rate in OD/HFD rats, in our study, may be related to the beginning of oxidative stress status verified in maternal erythrocytes, characterized by higher antioxidative enzyme activities (CAT and GSH-Px) in maternal erythrocytes of OD/HFD rats and an increased TBARS indicating lipoperoxidation. Oxidative stress is related to hyperglycemia, and it is also one of the main causes of alterations in the reproductive systems of women (Agarwal et al., 2012). Previous studies have reported that the balance between reactive oxygen species (ROS) and antioxidants greatly influences different phases of the reproductive activities in female mammalian animals (Al-Gubory et al., 2010; Wang et al., 2017), and oxidative stress conditions may compromise the reproduction and fertility (Agarwal & Allamaneni, 2004; Agarwal et al., 2012). The beginning of oxidative stress status in maternal erythrocytes can be a signal that organs, like the pancreas and ovaries, also present with an unbalanced redox system, and initial changes in this redox status are seen in the red blood cells of OD/HFD rats in our study might be related to the lower fertility and implantation rates as well. Unlike the liver, which presented the high activity of antioxidative enzymes and no alterations in MDA levels in our study, redox status during pregnancy varies among different organs and depends on the local regulation. The pancreas has a lower antioxidant defense and, consequently, a greater susceptibility of beta (β)-cells to oxidative stress (Lenzen et al.,

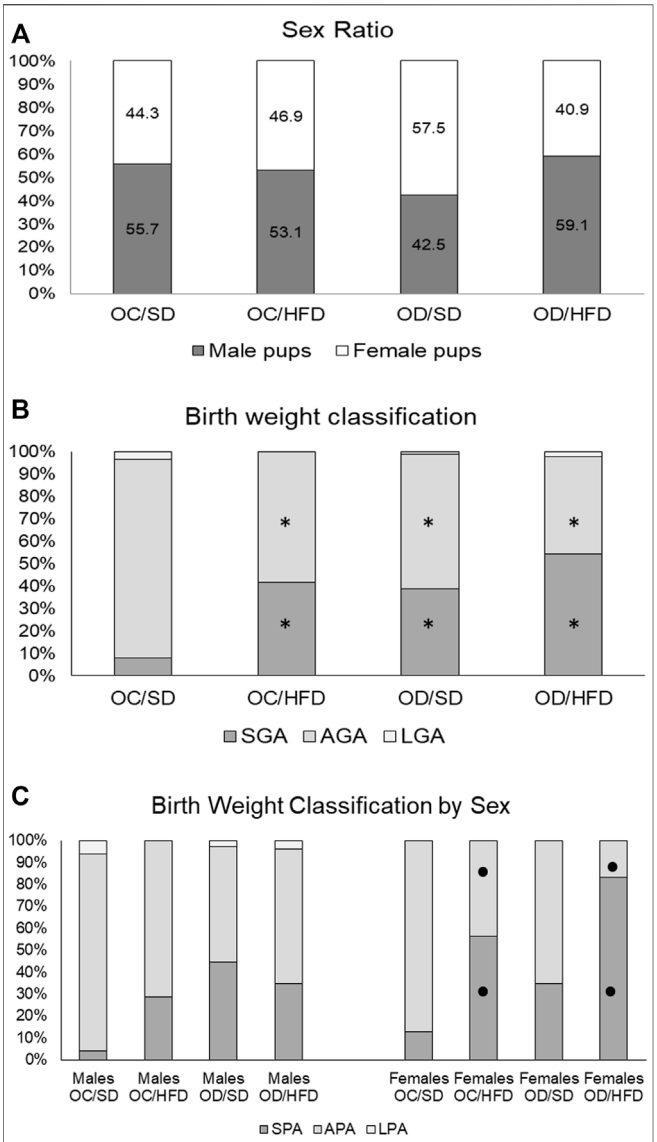

FIGURE 6 | (A) Sex ratio, **(B)** birth weight classification, and **(C)** birth weight classification by sex from OC, control offspring; and OD, diabetic offspring that received SD, standard diet or HFD, high-fat diet from weaning. Values are expressed in percentages. Legend: SPA—small fetuses for gestational age, APA—adequate fetuses for gestational age, and LPA—large fetuses for gestational age. *$p < 0.05$—compared to OC/SD group (Chi-square test) and •$p < 0.05$—between male and female pups from the same group (Chi-square test).

1996). Thus, the maladaptation of the endocrine pancreas to pregnancy can affect maternal glucose and insulin concentrations as seen in OD/HFD rats and harm reproductive organs. Exposure to adverse intrauterine conditions can lead to permanent changes in the structure and function of major physiological systems in developing fetuses (Yao et al., 2021). In rodents, hyperglycemia interferes with the development of fetal female gonads by reducing the weight, diameter, and volume of the ovaries and reducing the number of ovarian follicles and follicular diameter in these offspring (Khaksar et al., 2013). Similarly, overnutrition correlates with poor oocyte development in humans (Larsen, 2015), and a high-fat diet impairs oocyte quality and embryo development in mice (Bermejo-Alvarez et al., 2012). However, to understand the possible mechanisms involved in decreased fertility rates observed in OD/HFD female rats, a study focused on morphologic and molecular alterations in the ovaries of these offspring is underway in our research group.

Despite low fertility rates, when considering only the female rats that reached full-term pregnancy, the indirect parameter evaluated to study the delivered oocyte number-—the number of corpora lutea—presented no difference among experimental groups, showing no ovulation changes in this period. Nevertheless, OD/HFD rats showed a decrease in the number of implantations and live fetuses and higher rates of embryonic losses before implantation. Studies with early embryos (from two cells to blastocyst) in culture with high mean glucose concentration (Fraser et al., 2007) or *in vivo* study using hyperglycemic mother rats to evaluate their pre-embryos (Bueno et al., 2014) showed damages caused by hyperglycemic exposure during early embryonic development. This led to reduced cell numbers and increased apoptosis rates, which caused developmental delay and affected the success of embryonic implantation. Then, the lower number of implantations and live fetuses confirmed in the OD/HFD group might be related to embryonic complications in the early development, leading to higher percentages of preimplantation losses, which contributed to lower litter weight, compromising especially the female fetus growth.

Our results about glycemic metabolism surprisingly showed that the OD/HFD pregnant group presented no difference in insulin sensitivity, but the OD/SD rats had a decreased insulin sensitivity, being both groups performed by the quantitative insulin sensitivity check index (QUICKI). According to Katz et al. (2000), QUICKI was developed as an alternative method for glucose clamps. This method presented the best overall linear correlation with the gold standard clamp measurement, and it was more precise than the homeostasis model assessment of insulin resistance (HOMA-IR). Nevertheless, the authors disclose that QUICKI has limitations as the difficulty in applying it to type 1 diabetic individuals (without endogenous insulin secretion) or in patients with type 1 or type 2 decompensated diabetes (Katz et al., 2000). Considering the diabetic offspring, the OD/SD and OD/HFD rats had higher fasting glucose levels and AUC, confirming intolerance glucose status and higher insulin concentrations, confirming insulin resistance. Another study from our research group with control virgin female pups (OC) and diabetic offspring (OD) that received a standard diet (SD) or high-fat diet (HFD) from weaning until 120 days of life showed the presence of glucose intolerance in the OC/HFD, OD/SD, and OD/HFD rats, an increase in insulin synthesis, and the presence of insulin resistance before pregnancy on OC/HFD and OD/SD rats (Paula et al., 2021). The apparent conflicting result might indicate that these animals possibly

maintained an adequate adaptive pancreatic response in pregnancy, ameliorating the glucose intolerance presented before pregnancy. Normal pregnancy is associated with increased insulin resistance as a metabolic adaptation to the nutritional demands of the placenta and fetus. Insulin resistance is normally compensated by an adaptive increase in pancreatic β-cell mass together with enhanced glucose-stimulated insulin release (Moyce and Dolinsky, 2018). Studies have shown an increase in the number of cells and/or size of the pancreatic islet to compensate for the physiological demands of pregnancy (Fujimoto and Polonsky, 2009). The increased β-cell mass depends on a combination of the proliferation of existing β-cells, hypertrophic expansion, differentiation of resident progenitor β-cells, or islet cell transdifferentiation accompanied by a temporary decrease in apoptosis (Butler et al., 2010; Ernst et al., 2011; Bonner-Weir et al., 2012; Moyce and Dolinsky, 2018; Szlapinski and Hill, 2021). Thus, an overview of our results suggests the loss of an adequate adaptation of β-cells in OD/HFD rats during pregnancy, suggesting that QUICKI was not a good index in these cases.

The crosstalk among the maternal pancreas, placenta, and peripheral tissues is essential, and adiponectin and leptin are hormones involved in this signaling. The intrauterine exposure to hyperglycemia and placental methylation of leptin and adiponectin might lead to the development of leptin and insulin resistance and alter appetite circuits leading to postnatal hyperphagia, decreased satiety, and subsequent development of metabolic syndrome (Greco et al., 2019). However, our results showed no alterations in leptin concentration and food consumption related to total energy intake, showing no alterations in these circuits. The data about body weight gain and energy intake are controversial in several studies using different types of high-fat diets. According to the high-fat diet characteristics, overeating is induced, which may work together to promote the storage of dietary fat (Schack-Nielsen et al., 2010; Hull et al., 2011; Donovan et al., 2013). Besides, only female pups coming from control dams fed with a high-fat diet showed higher relative fat weight at term pregnancy, but it had no influence on adiponectin and leptin levels in maternal serum in the OC/HFD, OD/SD, and OD/HFD, corroborating Desai et al. (2014). There were no reported changes in the leptin and adiponectin concentrations measured in the placental tissue. Despite these results, the fetuses from OD/SD and OD/HFD rats had increased leptin serum levels, and these data do not corroborate the literature. Clinical studies have been addressing that leptin levels in the umbilical cord and newborns are positively correlated to birth weight and adiposity (Misra & Trudeau, 2011; Misra et al., 2013; Josefson et al., 2014; Ökdemir et al., 2018; Stefaniak et al., 2019). However, our results showed lower birth weight (SGA fetuses) and hyperleptinemia in the second generation of diabetic rats, which was not what we expected. These conflicting data observed in our rat model can be related to the low-fat percentage at birth in these newborns. Leptin plays an important role in controlling satiety and is an indicator of body fat mass. It is important to consider that this peptide may be involved with fetal growth in the third trimester when human fetal fat is deposited (Desai et al., 2014). Thus, in humans, the newborn fat depot occurs in the intrauterine milieu, however, in rats, this takes place after birth (Herrera and Amusquivar, 2000), which might have influenced the lower birth weight in our laboratory animals in this experiment. Additionally, higher serum leptin levels at birth should predispose to increased blood pressure in adult rats since leptin is well known to be a relevant marker and mediator of vascular dysfunction and hypertension (Gonzalez et al., 2013; Schinzari et al., 2013).

The classification of birth weight showed a significant increase in the proportion of small fetuses for gestational age and a decrease in the proportion of fetuses with adequate weight for gestational age in all groups when compared to control. In humans, high maternal body mass index (BMI) is associated with fetal overgrowth and macrosomia, and an increased risk of premature birth, instead maternal underweight increases the risk of low birth weight and small fetuses for gestational age (Liu et al., 2019). Regardless of this controversial finding, adequate placental function is directly responsible for fetal growth. Our study verified impaired placental efficiency in OD/HFD rats. The placenta plays a role underlying fetal programming and, therefore, is in part related to the origin of the development of health and disease (DOHaD). Both maternal glucose and nutrients from the high-fat diet are transmitted from the maternal to the fetal compartment through the placenta. This dynamic process might lead to changes in long-term health and disease outcomes in the first generation. In addition, the second generation might also be affected and may undergo reprogramming during the embryonic period in the primordial germ cells, which contributes to genetic and epigenetic information in the second generation (Dunn et al., 2011).

Our study showed that fetal restriction was predominant in female fetuses from both groups of dams fed a high-fat diet. The sex of the embryo affects the size of both the fetus and the placenta and the ability of the placenta to respond to adverse stimuli (Clifton, 2010; Mao et al., 2010; Gabory et al., 2013). In our study, this suggests that a high-fat diet interferes differently with male and female placental tissue, and thus, the differences in how male and female placentas cope with stressful conditions indicate that this tissue should also be taken into account if we want to understand how it contributes to health and disease later in life.

In conclusion, our findings show that fetal programming caused by maternal diabetes or lifelong high-fat diet consumption, in isolation, leads to similar repercussions in pregnant rats. In addition, the association of both conditions was responsible for glucose intolerance and oxidative stress in the first generation and increased fetal leptin levels in the second generation. Thus, our findings show both the F1 and F2 generations were negatively affected after the maternal hyperglycemic intrauterine environment and exposure to a high-fat diet from weaning until the end of pregnancy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for the Use of Animals of Botucatu Medical School, which approved all the methods adopted in this study (Protocol CEUA Number: 1218/2017).

AUTHOR CONTRIBUTIONS

All authors who have reviewed the manuscript agree with its contents and consent to its publication and that no other person has satisfied the criteria for authorship. We further confirm that all of us have approved the order of authors listed in the manuscript. YS, VP, and RM-S performed experiments; YS and DD drafted the manuscript and analyzed the data; JC performed statistical analysis; JC, YS, FG, GV, and DD interpreted the statistical data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.818621/full#supplementary-material

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Maternal and Offspring Sugar Consumption Increases Perigonadal Adipose Tissue Hypertrophy and Negatively Affects the Testis Histological Organization in Adult Rats

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Sugar intake has been associated with the development of male reproductive pathologies because of the increase and dysfunction in different adipose tissue depots. The establishment of these dysfunctions in the early stages of development is unknown. We evaluated the effect of maternal (pregnancy and lactation) and male offspring (from weaning to adulthood) consumption of 5% sucrose on perigonadal adipose tissue (PAT) and testis in adulthood. Moreover, two rat groups were compared, both including pregnant and lactating females: Control (C—drinking tap water) and sugar (S—consuming 5% sucrose solution). From weaning to adulthood with male offspring, four subgroups were formed: Control Mother → Control and Sugar offspring (CC, CS) and Sugar Mother → Control and Sugar offspring (SC, SS). At 120 postnatal days, the testes and PAT were collected and morphologically described. Furthermore, we quantified the number and cross-sectional area of perigonadal adipocytes and their distribution. We found that the males from SC and SS groups showed high PAT weight ($p < 0.005$), a high number ($p < 0.05$), and a relative frequency of large adipocytes ($p < 0.05$), establishing these results during gestational and lactation stages, and enhancing in adulthood since postnatal diet and its interaction. More macrophages, mast cells, and Leydig cells were observed in the interstitial space of the testis for the CS, SC, and SS groups, concluding that consumption of a high-carbohydrate maternal diet, program hypertrophy processes in adult PAT, developing and enhancing with sugar consumption during postnatal life. Furthermore, they are associated with inflammatory processes within the interstitial space of the testis.

Keywords: maternal programming, perigonadal adipose tissue, hypertrophy, testis, high sugar intake

Abbreviations: BMI, body mass index; BW, body weight; C, control; CC, control-control; CS, control-sucrose; CSA, cross-sectional area; HTB, blood–testicular barrier; MD, maternal diet; PAT, perigonadal adipose tissue; PNd, postnatal days; PND: postnatal diet; S, sucrose; SC, sucrose-control; SS, sucrose-sucrose; WAT, white adipose tissue.

INTRODUCTION

The caloric intake is closely related to diets rich in simple and rapidly assimilated sugars, such as glucose, fructose, and sucrose (Murphy and Johnson, 2003). Although the daily recommendation is 5%–10% of carbohydrates from the total energy intake in adults and children (Organization for Economic Co-operation and Development (OECD), 2017), the worldwide consumption of sugars has increased from 169 to almost 180 million metric tons (Murphy and Johnson, 2003; United States Department of Agriculture (USDA), 2020). This increase in the sugar consumption is present from gestation (Gamba et al., 2019; Casas et al., 2020) and breastfeeding (Zou et al., 2012), continuing throughout childhood (Dubois et al., 2007; De León-Ramírez et al., 2021) and adult life (Kumar et al., 2014). During gestation and breastfeeding, nutrition through the maternal diet plays a critical role (Cervantes-Rodríguez et al., 2014; Nicolás-Toledo et al., 2018; Pedrana et al., 2020), it is already well known that imbalances in the adequate consumption of macronutrients, such as proteins (Pedrana et al., 2020) and carbohydrates (Casas et al., 2020), negatively affect the development and maturation of different organs in later stages (Cervantes-Rodríguez et al., 2014; Nicolás-Toledo et al., 2018; Corona-Quintanilla et al., Online ahead of print) because both the glucose and fructose cross the placenta (Holmberg et al., 1956) and fetal development depends on transport of glucose through the mother's blood (Regnault et al., 2013). These programmed or established changes during the fetal/embryonic stage that can cause diseases in adulthood are known as the theory of the origin and development of diseases (Barker, 1990).

Both high-calorie diets (Jastrzębska et al., 2014; De León-Ramírez et al., 2021) and a BMI > 25 kg/m^2 (overweight and obesity) (Organization for Economic Co-operation and Development (OECD), 2017; Morales et al., 2014) is associated with male reproductive tract diseases related to some metabolic disorders (metabolic syndrome, insulin resistance, and dyslipidemias) (Nicolás-Toledo et al., 2018; De León-Ramírez et al., 2021). In animal models, high-carbohydrate diets have been established depending on the type and amount of mono, di, or polysaccharide used (Rodríguez-Correa et al., 2020). Also, it has already been described that consumption of these diets during pregnancy and postnatal stages can affect the function of the striated muscles associated with copulation (Corona-Quintanilla et al., Online ahead of print) and the expression of factors related to fat, such as the insulin receptor and the development of spermatogonias (Mao et al., 2018). In addition, postnatal consumption negatively affects testicular histology, injuring the intra and extra tubular epithelium (De León-Ramírez et al., 2021).

In mammals such as humans and rats, metabolic relationship between the testis and white adipose tissue (WAT) that surrounds it, better known as perigonadal adipose tissue (PAT), has not yet been clarified (Bjørndal et al., 2011; Luong et al., 2019) and even less the effect of high-carbohydrate diets on communication between both organs, especially if we consider that one of the main targets of metabolic pathologies associated with reproductive tract is adipose tissue, which is particularly vulnerable to changes in nutrition (Bibee et al., 2011).

Considering that limits of the perimeter of adipocytes observed under a histological slide resemble a polygonal mosaic whose vertices point outwards, like a Poisson–Voronoi diagram, the gamma distribution has been used as a statistical model proposal to characterize the adipocyte size distribution (Ibáñez et al., 2018). In this report, measures associated with the spread and shape of the data distribution under the assumption of gamma distribution are used to characterize the size distribution of perigonadal adipocytes and to determine, based on the theory of the developmental origins of the health and disease, its causal relationship with maternal consumption of sucrose and postweaning in male offspring.

The development of alterations in the male reproductive tract has been associated with sugar consumption and metabolic disorders such as for overweight and obesity (Martini et al., 2010; Sadeghi-Bazargani et al., 2013). The progress of these diseases is characterized by an abnormal or excessive accumulation of lipids in the adipocytes of different deposits of WAT (Cinti et al., 2019), as a result of the consumption of high-calorie diets (França et al., 2020; Oliveira et al., 2020). The PAT is one of its largest fat deposits (Berry et al., 2016) and it is the most important for the testis since its extraction in one or both gonads inhibits spermatogenesis (Chu et al., 2010). Although the joint consumption of carbohydrates and fats during pregnancy and postnatal life has adverse effects on the development of germ, Sertoli, and Leydig cells in mice (Mao et al., 2018), and the effect on PAT and testicular epithelium specifically by chronic consumption of simple sugars through maternal diet and throughout postnatal life is still unknown. For this, our objective was to determine the effect of 5% sucrose consumption in maternal (gestation and breastfeeding) and postnatal (childhood and adulthood) stages on the testis and PAT in adult male rats. We hypothesize that maternal consumption of sucrose may program the development of PAT hypertrophy, as well as histological alterations in the testes of male offspring; and this programming could be maintained and enhanced with the consumption of the same disaccharide from early childhood (weaning) to adulthood.

MATERIALS AND METHODS

Animals

All procedures applied to animals were approved by the Bioethics Committee the Centro Tlaxcala de Biología de la Conducta from Universidad Autónoma de Tlaxcala, following the Mexican guide for animal care (NOM-062-Z00-1999, Mexico). Twelve pregnant female (14-week-old) Wistar rats (*Rattus norvegicus*), weighing 220–240 g, were housed individually in polypropylene cages (37 cm × 27 cm × 16 cm) and maintained in controlled rooms with temperatures of 23 ± 1°C and a 12-h light–dark cycle (with the light off from 08:00 h to 20:00 h).

Experimental Design

The details of the experimental design used in this study have been published previously (Corona-Quintanilla et al., Online ahead of print). Primiparous female rats were paired with

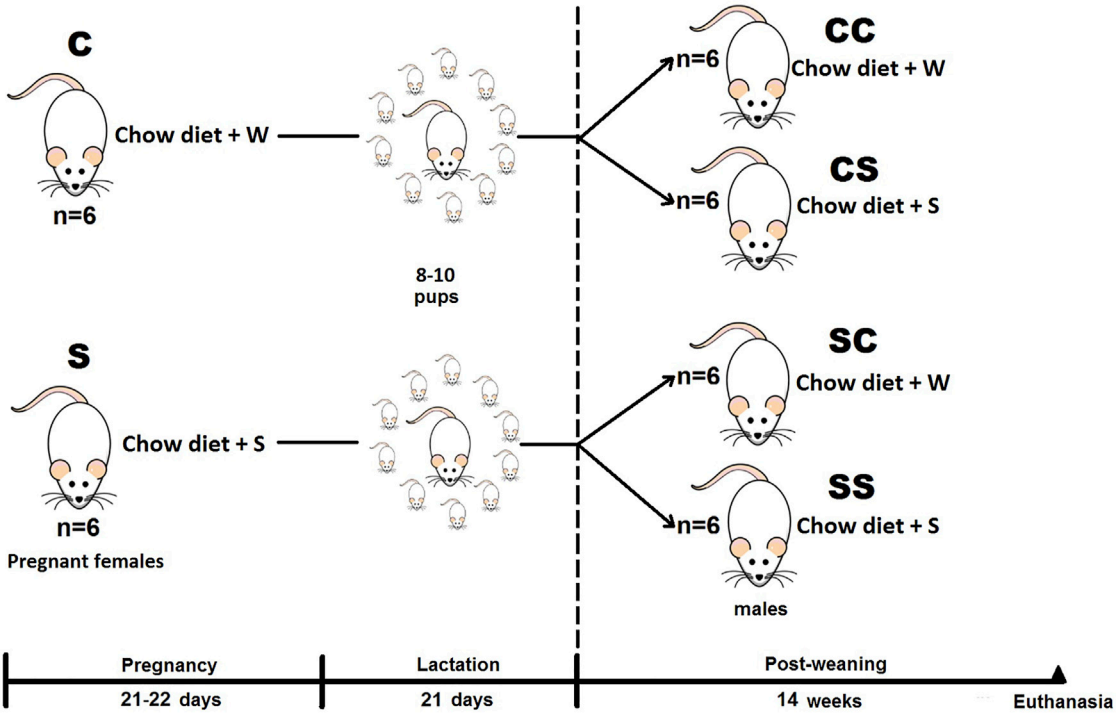

FIGURE 1 | Experimental design. Groups of pregnant and breastfeeding females, and from male rats from weaning to adulthood, fed with a standard diet and tap water (W) without (C) and added 5% sucrose (S).

males of proven fertility. The female was individually placed in polypropylene boxes, considering the day of mating as day 0 of gestation. During pregnancy and breastfeeding, the females were randomly assigned into two groups: a control group (C), which drank unadulterated tap water, and an experimental group that was provided a 5% sucrose solution (S) to drink. Postpartum, the litters were adjusted to 8–10 pups and from postnatal day 22–120, and the male offspring were in pairs or trios placed in polypropylene boxes and fed with either tap water or 5% sucrose according to the assigned experimental group: control mother-control offspring (CC), control mother-sucrose offspring (CS), sucrose mother-control offspring (SC), and sucrose mother-sucrose offspring (SS) (**Figure 1**).

Dietary Protocol

All rats were fed daily with Chow-5001 standard diet (LabDiet 5001-Laboratory Rodent Diet, St. Louis, MO, United States) and tap water *ad libitum*, and 5% sucrose was diluted in the tap water for the S groups. The conditions of maintenance and care of the animals were previously established (Corona-Quintanilla et al., Online ahead of print). Notably, this amount of sugar added to the diet does not decrease the energy intake of the equivalent to 15% protein, as we have previously reported (Cervantes-Rodríguez et al., 2014; Corona-Quintanilla et al., Online ahead of print), because the consumption of this nutrient can be considered deficient when it is very low (3% and 5%) and moderately low (8% and 12%), or adequate when equal to 15% and 20% (Moro et al., 2021). This study aims to investigate the effect of sugar consumption without the interference of protein deficiency.

Water and food consumption and body weight were measured daily at 9:30 h during pregnancy, breastfeeding, and adulthood (offspring) (Corona-Quintanilla et al., Online ahead of print) to calculate weight gain, consumption of carbohydrates, lipids, and proteins, as well as their respective individual and total caloric intake. These parameters were daily evaluated according to the nutritional description of the Chow 5001 food.

Obtaining Tissues

Before euthanasia, males from 120 postnatal days (PNd) fasted for 12 h. Male rats were deeply over-anesthetized with sodium pentobarbital (50 mg/kg BW) and decapitated using a rodent guillotine device (Harvard Apparatus, Holliston, MA). After decapitation, animals were placed in a supine position and made a longitudinal incision on the abdominal cavity. Both testes with the epididymis and PAT were harvested and weighed (g tissue/100 g BW) (De León-Ramírez et al., 2021).

Morphometric Analysis

At birth, pup weight, body length (from the tip of the nose to base of tail), heat diameter, abdominal diameter, and anogenital distance were measured using calipers. Left testes were removed, fixed, and dehydrated according to the procedure established by De León-Ramírez et al. (2021). They were embedded in Paraplast Plus (Sigma-Aldrich. St. Lois MO, United States), cut at 7 μm, and stained with hematoxylin and

TABLE 1 | Growth and metabolic parameters of adult females that consumed simple (C) and 5% sucrose (S) water during gestation and breastfeeding.

	Pregnant females			
	C	S	Variation (%)	p values
Pregnancy initial body weight (1 GD) (g/d/100 g BW)	232 ± 3.1	240 ± 3.5	3.4[a]	0.1235
Pregnancy final weight (21 GD) (g/d/100 g BW)	366 ± 2.0	382 ± 6.3	4.4[a]	0.0342
Pregnancy weight gain (1–21 GD) (g/d/100 g BW)	134 ± 3.2	142 ± 6.2	6.2[a]	0.2613
Breastfeeding initial body weight (1 PNd) (g/d/100 g BW)	274 ± 6.5	270 ± 7.3	1.6[b]	0.6506
Breastfeeding final body weight (21 PNd) (g/d/100 g BW)	308 ± 10.4	300 ± 5.5	3.6[b]	0.5150
Breastfeeding weight gain (1–21 PNd) (g/d/100 g BW)	38.8 ± 2.5	34.7 ± 4.9	10.6[b]	0.4728
Food consumption (g/d/100 g BW)	8.7 ± 0.1	6.7 ± 0.3	23.1[b]	0.0002
Water consumption (ml/d/100 g BW)	21.6 ± 1.2	42.8 ± 6.7	98.0[a]	0.0109
Carbohydrate consumption (g/100 g BW)	88.1 ± 2.7	108.6 ± 4.8	23.2[a]	0.0045
Carbohydrate energy intake (kcal/100 g BW)	352 ± 11.1	434 ± 19.5	23.2[a]	0.0045
Lipid consumption (g/100 g BW)	18.7 ± 0.3	14.4 ± 0.6	23.1[b]	0.0002
Lipid energy intake (kcal/100 g BW)	169 ± 3.3	130 ± 6.1	23.0[b]	0.0002
Protein consumption (g/100 g BW)	41.9 ± 0.8	32.2 ± 1.5	23.0[b]	0.0002
Protein energy intake (kcal/100 g BW	167 ± 3.3	129 ± 6.1	23.1[b]	0.0002
Total energy intake (kcal/100 g BW)	689 ± 14.9	693 ± 18.9	0.6[a]	0.8613

The mean values ± SEM (n = 6) are observed.
[a]Increase in the percentage variation of metabolic parameters respect control group.
[b]Decrease in the percentage variation of metabolic parameters respect control group.
GD, gestational day; PNd, postnatal day, BW, body weight.

eosin (H&E) (De León-Ramírez et al., 2021). Sections were visualized and photographed using a light microscope (Zeiss Axio Imager A1) at ×2.5, ×4, ×10, and ×40. For the cross-sectional area (CSA) and number of transversally cut seminiferous tubules quantification, reconstructions of microphotographs were conducted at ×4 using the Adobe Illustrator CS5 program. The analysis of histological characteristics of the seminiferous epithelium was performed according to Morales et al. (2014). After excision, random samples of left gonadal fat were fixed, dehydrated, cut, and stained according to the protocol of Cervantes-Rodríguez et al. (2014). Slides were collected forming a series of 10 sections and discarding 10 until 30 sections were obtained per sample. Adipocytes were observed using an optical microscope (Axio Imager A1, Zeiss) coupled to an Olympus digital camera.

The number and CSA of adipocytes were measured with the support of a grid in the central zone of the photomicrographs obtained at ×10 and ×40 during the reconstruction of the PAT sections photographed with Axiovision 4.8 software (Carl Zeiss MicroImaging, Inc.). Relative frequency histograms were plotted with 200 μm^2 CSA intervals (i.e., 0–200, 201–400, and 401–600 μm^2) up to the maximum observed CSA interval.

Estimation of Small and Large Adipocyte Proportions

The cutoff points for small, medium, and large adipocytes were defined based on the 25th and 75th percentiles of the relative distribution of the CC group and analysis by non-parametric comparisons.

Statistical Analyses

The sample size in each group was six rats from different litters. Following the normality of the data (with a frequency distribution histogram), the comparison of groups of mothers during pregnancy and breastfeeding was done by Student's t test.

Two-way ANOVA was used to compare data of body weight, weight gain, food intake, water consumption, testicular, and PAT parameters (number, weight, and CSA of seminiferous tubules and adipocytes, as well as adipocytes size—small, medium, and large proportion) in male offspring for all groups by the combined effects of period diet and sucrose consumption. Period diet (maternal consumption: gestation/breastfeeding; postnatal: childhood/puberty/adulthood) was considered as the first independent variable and sugar intake as the second independent variable. Where ANOVA indicated a significant ($p < 0.05$) effect of treatments, a *post hoc* test was carried out using the Tuckey correction to determine significant differences.

The CSA distribution of adipocytes and seminiferous tubules was analyzed using frequency histograms and subsequently Fisher's test by X^2 was used to compare the percentage of area according to size bin.

GraphPad Prism Version 6.0 program for Windows was used and all variables measured were expressed as means ± SEM. Significant differences were considered with a $p \leq 0.05$ for all cases.

RESULTS

Growth and Nutritional Consumption of Pregnant Mothers

Differences in weight and consumption of water and food are shown in the **Table 1**. During pregnancy, there were no differences between the C and S groups in the body weight at beginning of gestation nor in weight gain, but 5% sucrose intake affected the weight, showed at the end of pregnancy (**Table 1**). Throughout breastfeeding, there were no differences in initial, final, or weight gain.

TABLE 2 | Weight, morphometric measurements, and water and food intake obtained at the birth, breastfeeding (from 1 to 21 PNd), and postnatal stage (22 to 120 PNd) of male offspring that consumed sucrose at 5% (CS and SS) and simple (CC and SC) water and whose mothers during gestation and breastfeeding consumed the same diets. The effect of diets during the pre (MD) and postnatal (PDN) periods and their interaction are shown.

	Pups		
	C	S	p-value
Weight 1 PNd (g)	7.1 ± 0.3	7.5 ± 0.3	0.3819
Weight 21 PNd (g)	38.2 ± 2.5	39.0 ± 1.5	0.7929
Weight gain 1 to 21 PNd (g)	31.0 ± 2.4	31.5 ± 1.7	0.8787
Length (mm)	49.4 ± 0.8	51.9 ± 0.9	0.8027
Head circumference (mm)	12.0 ± 0.3	11.7 ± 0.2	0.5083
Abdominal circumference (mm)	14.8 ± 0.9	15.4 ± 0.4	0.5440
Anogenital distance (mm)	3.4 ± 0.1	3.4 ± 0.1	0.9071

	Offspring male groups				p-value		
	CC	CS	SC	SS	MD	PND	MD × PND interaction
Average initial feed (21–28 PNd) intake (g/d/100 g BW)	17.3 ± 0.8	15.1 ± 0.2	17.0 ± 1.4	14.0 ± 0.5	0.3988	0.0085	0.6484
Average final feed (113–120 PNd) intake (g/d/100 g BW)	6.5^{a} ± 0.3	4.4^{b} ± 0.2	5.9^{a} ± 0.4	4.3^{b} ± 0.4	0.3824	< 0.0001	0.4984
Average total feed intake (g/d/100 g BW)	9.4^{a} ± 0.3	7.6^{bc} ± 0.1	9.0^{ac} ± 0.4	7.5^{b} ± 0.4	0.4539	0.0002	0.6058
Initial water (21–28 PNd) intake (ml/d/100 g BW)	37.6 ± 3.4	46.2 ± 5.0	34.1 ± 4.7	38.7 ± 2.7	0.1968	0.1243	0.6238
Final water (113–120 PNd) intake (ml/d/100 g BW)	15.0^{a} ± 1.2	30.5^{b} ± 2.8	13.1^{a} ± 1.9	29.4^{b} ± 4.1	0.6060	< 0.0001	0.8882
Total water intake (ml/d/100 g BW)	19.7^{a} ± 1.2	31.5^{b} ± 1.8	19.2^{a} ± 2.4	30.2^{b} ± 2.5	0.6564	< 0.0001	0.1079

Mean values ± SEM (n = 6) are observed. Superscript letters indicating differences between groups. Data analyzed with a two-way ANOVA and Tukey post hoc*. MD: Maternal diet, PND: postnatal diet, PNd: Postnatal day, BW: body weight.*

During maternal consumption period, females from the S group significantly increased their intake of both sugar water (98%) and total carbohydrates (by ~ 23%). Likewise the consumption of solid food (lipids and proteins) decreased ~ 23%; (**Table 1**). Respect to the protein consumption, it is important to mention that the caloric intake in the S group of dams was equivalent to 18.5%, while 24.1% was for the control group.

Morphometric Measurements of Pups at Birth

Male offspring from dams who consumed sucrose during pregnancy and breastfeeding did not show effects on their initial or final breastfeeding weight, length, head, and abdominal circumference, or anogenital distance (**Table 2**).

Growth and Nutritional Consumption of Males During Postnatal Stage

The sucrose intake did not modify the body weight (g) at 22 PNd (CC: 57 ± 2.2, CS: 64 ± 2.8, SC: 58 ± 3.2, and SS: 60 ± 4.1) at any life period (maternal consumption diet: $p = 0.5732$; postnatal diet: $p = 0.1960$; and interaction: $p = 0.4807$). There was an increase in the weight gain after 10 weeks in those groups that consumed sucrose at some period of their development, compared to the CC group (**Figure 2A**). This increase in the weight varied accordingly to the period of life in which sucrose was intake. Significant differences were found from 12 to 17 weeks since prenatal intake (CC vs. SC, **Figure 2B**), during the 7th week and from 12 to 17 weeks since postnatal consumption (CC vs. CS, **Figure 2C**), and from 13 to 17 weeks since consumption interaction in both periods (CS vs. SC and CC vs. SS; **Figures 2D,E**).

At 120 PNd, the weight (g) increased with sugar intake (CC: 379 ± 3.6, CS: 422 ± 3.7, SC: 401 ± 3.7, and SS: 415 ± 3.6) exhibiting significant differences between CC vs. CS ($p \leq 0.0001$), CC vs. SC ($p \leq 0.005$), CC vs. SS ($p \leq 0.0001$), and CS vs. SC ($p \leq 0.005$). A weight gain (g) was also observed from 22 to 120 PNd (CC: 321 ± 3.7, CS: 358 ± 5.3, SC: 343 ± 4.0, and SS: 355 ± 6.0), with significant differences between the groups CC vs. CS ($p \leq 0.05$), CC vs. SC ($p \leq 0.05$), and CC vs. SS ($p \leq 0.005$).

The postnatal sucrose intake had effect on both the body weight at 120 PNd ($p \leq 0.0001$) and weight gain ($p \leq 0.0001$) (**Figures 2A,C**). However, there was a significant effect by the interaction (**Figures 2D,E**) of maternal and postnatal diets (final weight: $p = 0.0005$ and weight gain: $p = 0.0160$). The effect of the postnatal diet was enhanced by the maternal diet (final weight, $p = 0.0596$ and weight gain, $p = 0.0740$).

All data related to water and food intake in male groups are presented in the **Table 2**.

The initial, final, and total food consumption at the postnatal period was significantly decreased only in the groups with 5% sucrose postweaning intake. At the beginning of this study, there were no differences in the food intake between the groups. However, at the final of treatment, the food intake (CC vs. CS $p \leq 0.005$; CC vs. SS $p \leq 0.005$; CS vs. SC $p \leq 0.05$; and SC vs. SS $p \leq 0.05$) and total consumption (CC vs. CS $p \leq 0.005$; CC vs. SC $p \leq 0.005$; and SC vs. SS $p \leq 0.05$) were different.

The initial water consumption was not affected by the maternal or postnatal sucrose intake, nor by the interaction (**Table 2**). However, in the postnatal stage, the final (CC vs. CS $p \leq 0.005$; CC vs. SS $p \leq 0.005$; CS vs. SC $p \leq 0.005$; and SC vs. SS $p \leq 0.005$) and total (CC vs. CS $p \leq 0.005$; CC vs. SS $p \leq 0.005$; CS vs. SC $p \leq 0.005$; and SC vs. SS $p \leq 0.005$) water consumption was significantly increased by 5% sucrose intake.

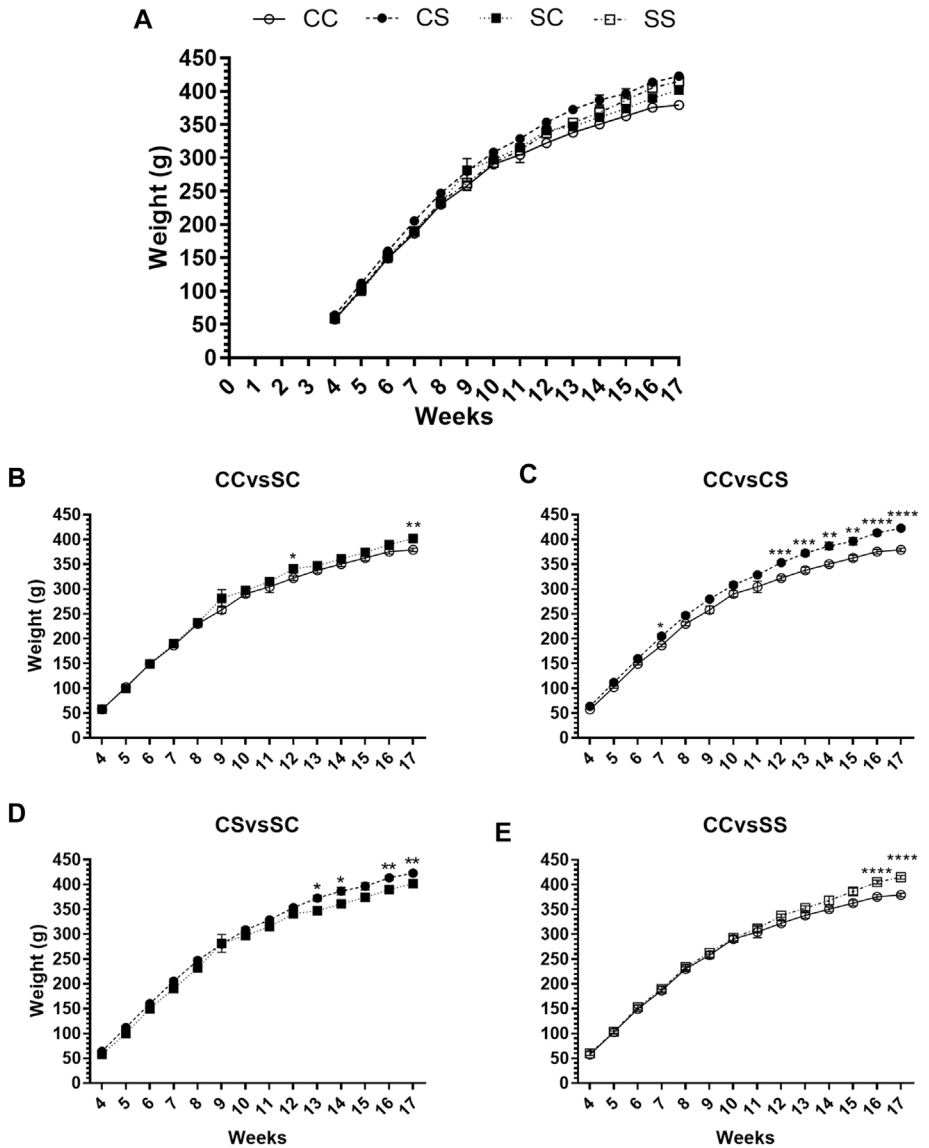

FIGURE 2 | Weekly weight gain **(A)** of CC (white circle and solid line), CS (black circle with dotted line), SC (black square with dotted line), and SS (white square with dotted line) male groups from weaning at 120 PNd. Comparisons per week between groups of males that presented differences due to maternal **(B)** or postnatal diet **(C)** and interaction **(D,E)**. Mean values ±SEM ($n = 6$) are shown. Data were analyzed with a two-way ANOVA and Tukey *post hoc*. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Data related to nutritional intake are presented in the **Table 3**. The decrease in the consumption of both proteins and lipids and their respective energy calculation in the CS and SS groups were due to consumed postnatal sucrose with extremely significant effect (corresponding to 49% of the variance in said variables), as well as significant differences were observed between groups. In the CS, SC, and SS groups, the decrease in the lipids and proteins was 19%, 4.5%, and 20%, respectively. Specifically, the equivalent of protein caloric intake in these groups ranged between 19% and 23% (CS: 19.5%; SC: 23.0%; and SS: 19.2%), compared to the CC group which was 24.1%.

The increase in the carbohydrate consumption was 15% and 12% in the CS and SS groups compared to the CC group. This increase (38.7% of the total variance) was associated with postnatal carbohydrate consumption and its energy calculation. A significant difference between groups was found only between the CS and SC groups. Both the total caloric intake and hematocrit level in adult males for all groups were unaffected by maternal and postnatal diets, or their interaction.

Morphometric Analysis of the Testis

Testis variables such as the relative weight, CSA, and number of seminiferous tubules were unaffected by maternal and postnatal diets or their interaction (**Table 3**). Relative distribution of the CSA of the seminiferous tubules was carried out (**Figure 3**). The seminiferous tubules had CSA ranging from 20 to 120 μm^2,

TABLE 3 | Sum of total consumption and energy intake of proteins, lipids, and carbohydrates from 4 to 17 weeks, and parameters testis in the groups of males that consumed sucrose at 5% (CS and SS) and simple (CC and SC) water during the postnatal stage (22 to 120 PNd) and whose mothers during pregnancy were fed the same diets. The effect of diets during the maternal (MD) and postnatal (PND) periods and their interaction are shown.

	Offspring male groups				p-value		
	CC	CS	SC	SS	MD	PND	MD × PND interaction
Total protein intake (g/d/100 g BW)	$31.5^{a} \pm 1.0$	$25.4^{bc} \pm 0.4$	$30.1^{ac} \pm 1.6$	$25.1^{b} \pm 1.4$	0.4840	0.0002	0.6446
Protein energy intake (kcal/d/100 g BW)	$126.1^{a} \pm 4.0$	$101.9^{bc} \pm 1.7$	$120.4^{ac} \pm 6.5$	$100.7^{b} \pm 5.6$	0.4837	0.0002	0.6453
Lipids intake (g/d/100 g BW)	$14.1^{a} \pm 0.4$	$11.4^{bc} \pm 0.2$	$13.4^{ac} \pm 0.7$	$11.2^{b} \pm 0.6$	0.4842	0.0002	0.6433
Lipids energy intake (kcal/d/100 g BW)	$127.0^{a} \pm 4.0$	$102.6^{bc} \pm 1.8$	$121.2^{ac} \pm 6.5$	$101.4^{b} \pm 5.7$	0.4836	0.0002	0.6448
Carbohydrates intake (g/d/100 g BW)	$64.2^{a} \pm 2.0$	$73.9^{ab} \pm 1.0$	$61.3^{ac} \pm 3.3$	$72.4^{a} \pm 4.1$	0.4515	0.0017	0.8078
Carbohydrates energy intake (kcal/d/100 g BW)	$257^{a} \pm 8.1$	$295^{ab} \pm 4.0$	$245^{ac} \pm 13.3$	$289^{a} \pm 16.4$	0.4519	0.0017	0.8075
Total energy intake (kcal/100 g BW)	510 ± 16.2	500 ± 5.6	487 ± 26.4	492 ± 27.3	0.4582	0.9076	0.7267
Hematocrit 120 PNd (%)	68.5 ± 1.7	66.3 ± 1.5	69.1 ± 0.5	62.1 ± 8.2	0.6843	0.2997	0.5780
Relative weight of testes (g)	0.48 ± 0.01	0.44 ± 0.01	0.47 ± 0.03	0.48 ± 0.02	0.6355	0.6143	0.3634
Number of sections counted of seminiferous tubules	468 ± 60.6	553 ± 51.4	489 ± 38.0	562 ± 56.6	0.7740	0.1416	0.9102
Seminiferous tubule cross-sectional area (CSA) (μm^2)	52.1 ± 3.3	45.9 ± 1.6	51.3 ± 2.8	47.1 ± 2.0	0.9548	0.0550	0.7061

Mean values ±SEM (n = 6) are observed. Superscript letters indicating differences between groups. Data analyzed with a two-way ANOVA and Tukey post hoc*. MD, Maternal diet, PND, postnatal diet, BW, body weight.*

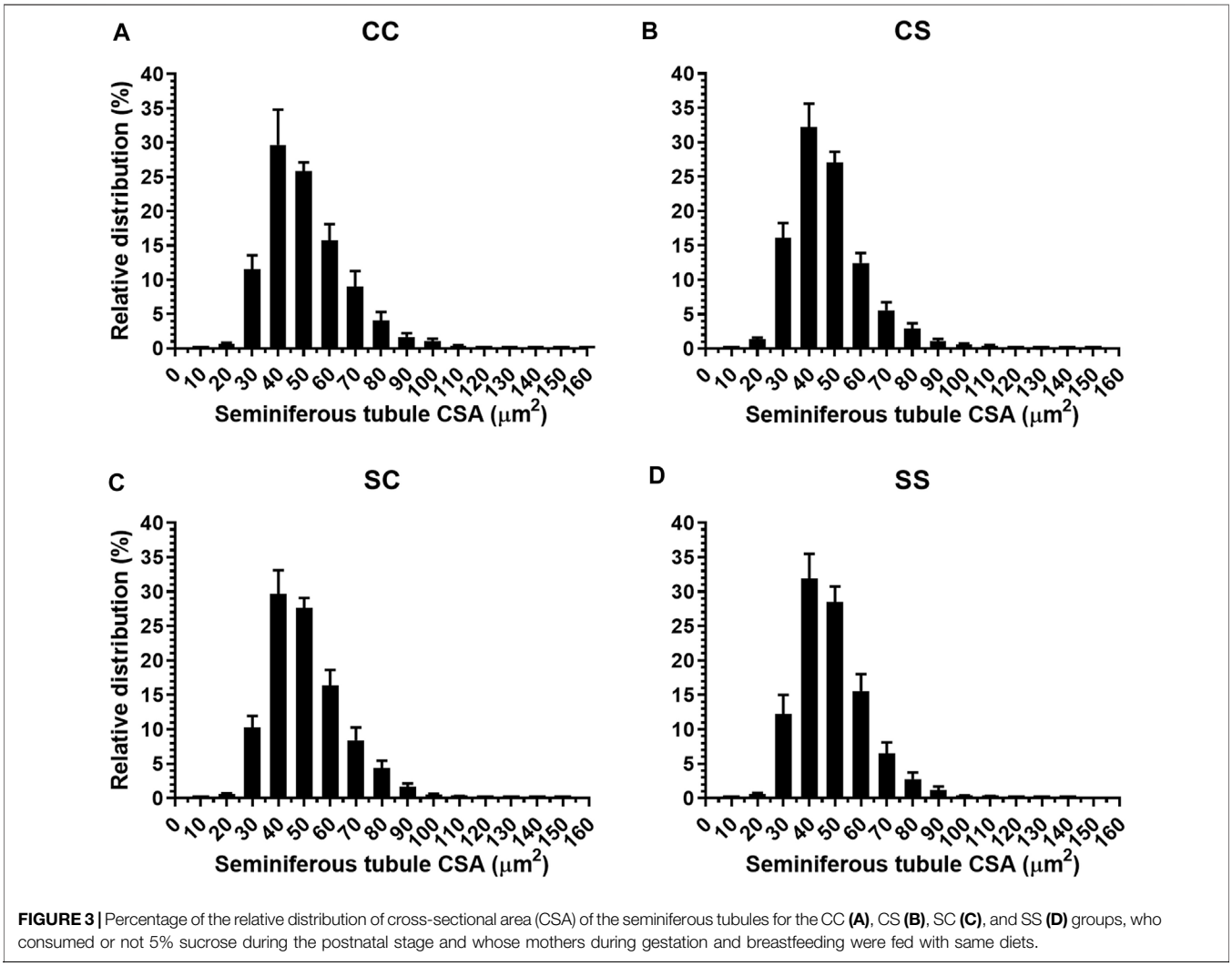

FIGURE 3 | Percentage of the relative distribution of cross-sectional area (CSA) of the seminiferous tubules for the CC **(A)**, CS **(B)**, SC **(C)**, and SS **(D)** groups, who consumed or not 5% sucrose during the postnatal stage and whose mothers during gestation and breastfeeding were fed with same diets.

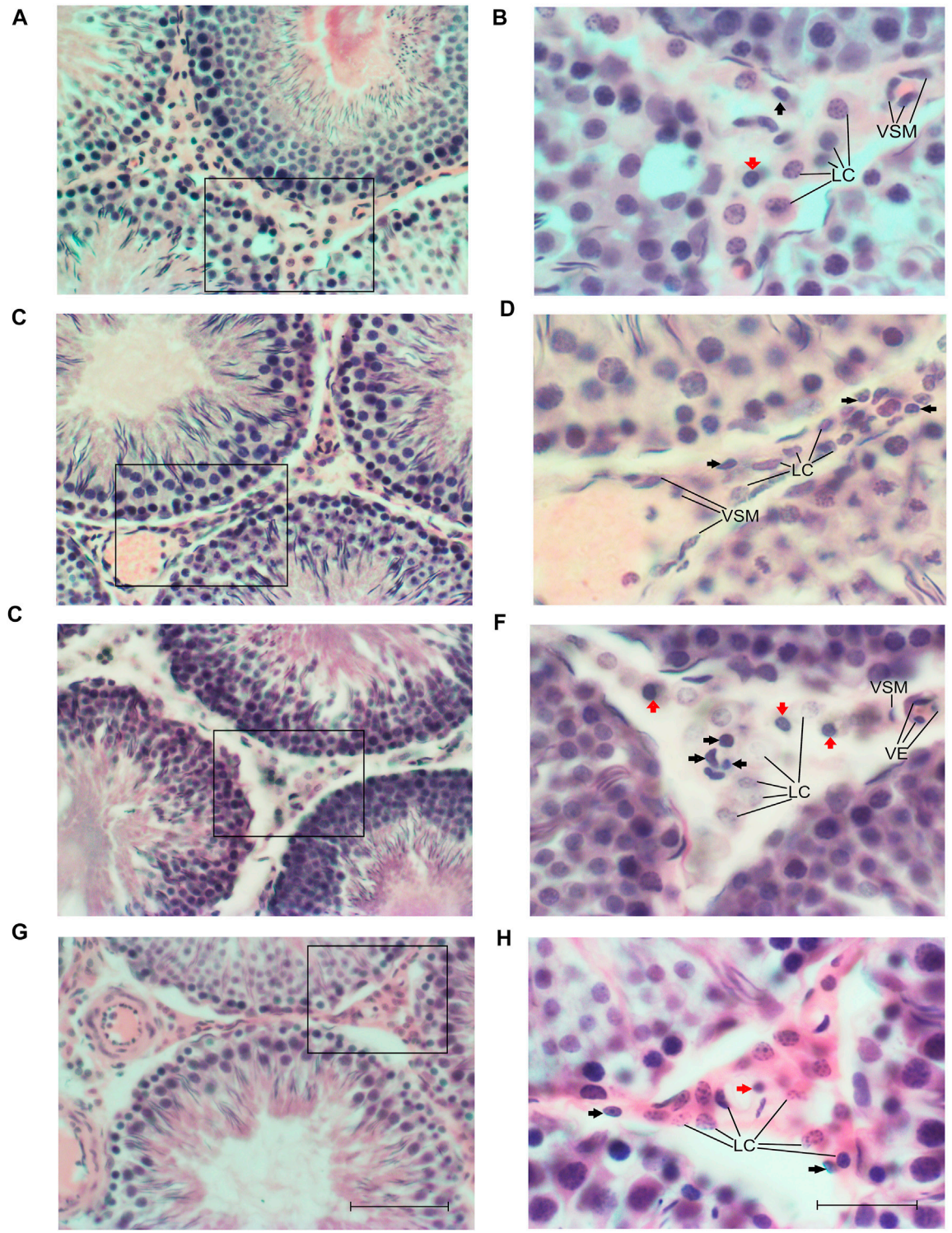

FIGURE 4 | Photomicrographs of the cross-sectional area (CSA) of testis stained with H&E at ×10 (scale bar = 100 μm) (a, c, e, and g) and ×40 (scale bar = 50 μm) **(B, D, F, H)** for the CC **(A,B)**, CS **(C,D)**, SC **(E,F)**, and SS **(G,H)** groups. Abbreviations: Interstitial space of the Leydig cells (LC), blood vessels (BV), vascular epithelium (EV), vascular smooth muscle (VSM) cells, macrophages (black arrows), and mast cells (red arrows).

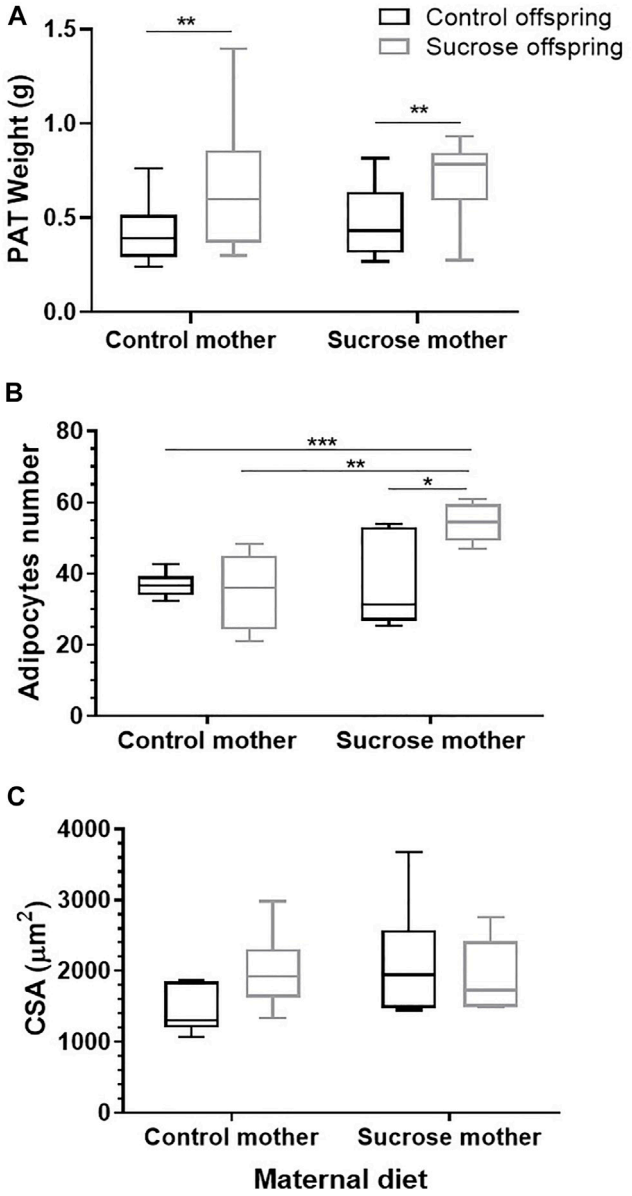

FIGURE 5 | Relative weight of TAP **(A)**, number **(B)**, and cross-sectional area (CSA) **(C)** of adipocytes of males that consumed 5% sucrose (gray box) and tap water (black box) during the postnatal period and whose mothers during pregnancy and breastfeeding were fed with same diets. Data were analyzed with a two-way ANOVA and Tukey *post hoc*. Significant differences are shown. *p < 0.05, **p < 0.01, and ***p < 0.001.

finding the highest percentage (more than 50%) among 40–59 μm^2, and presenting a Gaussian distribution around this interval. However, there were no differences between them (**Figure 3**).

No differences in the cell arrangement, typical presence of Sertoli cells in the basal area, germ cells (spermatogonia, spermatocytes, spermatids, and sperm), tunica propria (peritubular tissue of myoid cells) of each tubule, Leydig cells, and other populations of interstitial cells were found between groups (**Figures 4A–H**). However, a high number of interstitial cells, macrophages, and mast cells were observed in those groups that consumed sucrose at some stage of their development (**Figures 4A–H**).

Morphometric Analysis of Perigonadal Adipose Tissue

The relative weight of the PAT (**Figure 5A**) for the CS (0.6 g ± 0.1) and SS (0.7 g ± 0.1) groups was significantly increased in comparison with the CC (0.4 g ± 0.1) and SC (0.4 g ± 0.1) groups, having 20.2% of variation to the effect of 5% sucrose postnatal consumption ($p = 0.0044$). Neither the maternal diet ($p = 0.604$) nor the interaction ($p = 0.8774$) of diets between both periods had any significant effect on this variable. The number of adipocytes (**Figure 5B**) was significantly increased by the maternal diet ($p = 0.0189$), interaction ($p = 0.0214$), and postnatal diet ($p = 0.0528$). *Post hoc* tests showed significant differences between the SS (54 ± 2.4) and the CC (36 ± 1.4), and the CS (35 ± 4.7) and SC (37 ± 5.2) groups.

For the CSA average (μm) of adipocytes (**Figure 5C**), no differences were found between the CC group (1439 ± 138) and those who consumed sucrose in the maternal (SC: 2117 ± 336) and/or postnatal (CS: 1996 ± 225; SS:1917 ± 215) periods [maternal ($p = 0.2252$) and postnatal ($p = 0.4649$) diets or by their interaction ($p = 0.1291$)].

Frequency Distribution of Perigonadal Adipocytes

The relative frequency distribution of adipocytes with intervals of 200 units in the four groups of males (**Figure 6A**) presented a Gaussian-type distribution, loaded toward the extreme left, with a high proportion of adipocytes with CSA smaller than 1051 μm^2.

Only groups that consumed sucrose at some period of their life presented adipocyte proportions with CSA greater than 3,600 μm^2 (**Figures 6B–D**), especially the SC and SS groups (**Figures 6C,D**), whose proportion of adipocytes with CSA of 4,800–9,000 μm^2 was constant in almost all established intervals.

Comparisons were done between intervals of groups and showed that postnatal sucrose consumption decreased the proportion of adipocytes considered small (≤1,051 μm^2), increasing proportions for large adipocytes (≥1,789 μm^2) (**Figure 7**), being approximately 6% of adipocytes had CSA from 3,600 to 9,000 μm^2 (**Figures 6C,D**).

Comparisons with Fisher's test by X^2 between groups with intervals of 200 units were done, resulting in significant differences in adipocytes with CSA from 1001 to 1200 and 1201 to 1400 by postnatal diet (CC vs. CS, $p = 0.0400$). However, to properly distinguish the effect of diet in the two periods, one more comparison (data not plotted) was done with frequency intervals of 400 units. Resulting in a significant decrease in the proportion of CSA adipocytes from 801 to 1200 μm^2 ($p = 0.0281$) by maternal diet (CC vs. SC) and in those from 1,201 to 1,600 μm^2 ($p = 0.0414$) by postnatal diet (CC vs. CS). To corroborate these results, two comparisons were done with frequency intervals of 600 and 1200 μm^2 (histograms are not

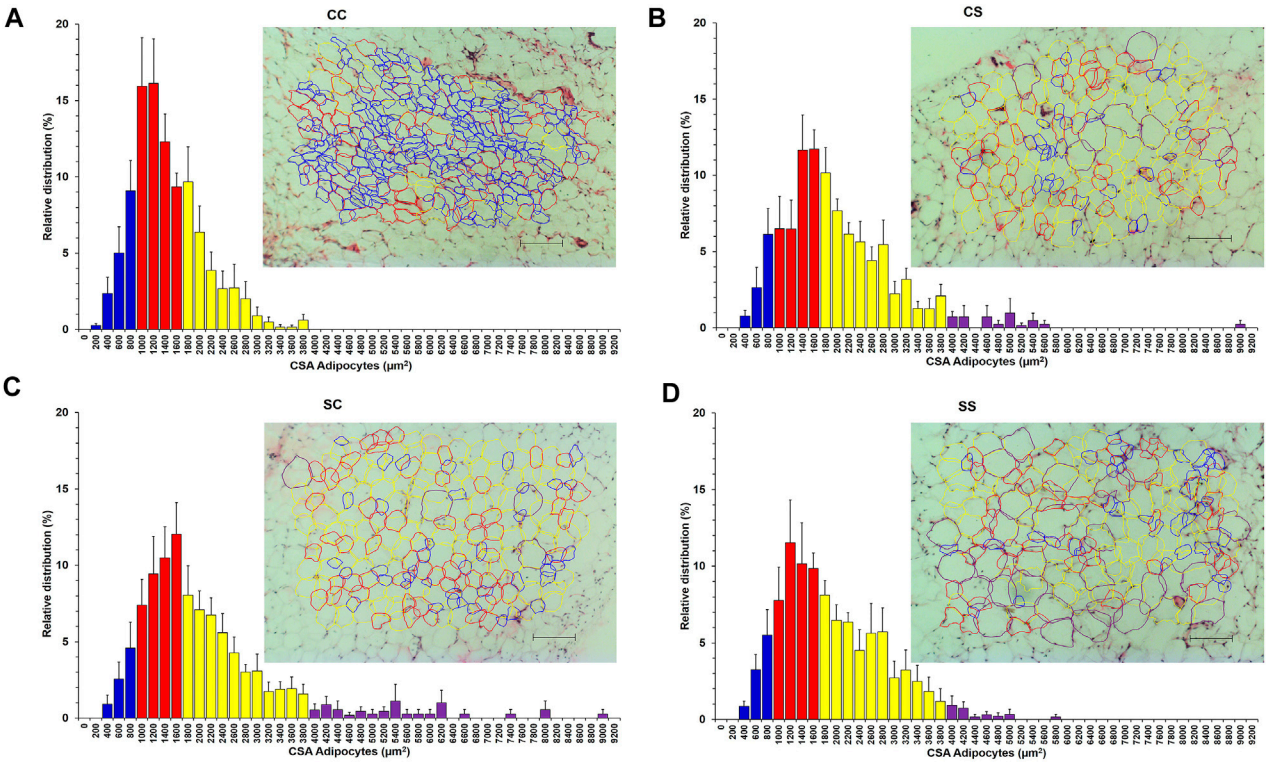

FIGURE 6 | Relative frequency distribution of adipocytes counted according to cross-sectional area (CSA) for the CC **(A)**, CS **(B)**, SC **(C)**, and SS **(D)** groups. Mean values ±SEM ($n = 6$) are found. Photomicrographs of perigonadal adipocytes of CC **(A)**, CS **(B)**, SC **(C)**, and SS **(D)** groups stained with H&E at ×10 (scale bar = 100 µm). The color of bars in graphics corresponds with the outline drawn of adipocytes. Blue (small, ≤1,051 µm^2), red (medium, from 1,052 to 1,788 µm^2), yellow (large, ≥1,789 µm^2), and purple (those with a CSA greater than 4,000 µm^2).

shown), which found the same pattern of differences in the range from 1,201 to 1,800 µm^2 (maternal diet, CC vs. SC: $p = 0.0178$; postnatal diet, CC vs. CS: $p = 0.0072$) and from 1,201 to 2,400 µm^2 (maternal diet, CC vs. SC: $p = 0.0005$, postnatal diet, CC vs. CS: $p = 0.0022$).

Estimation of Small and Large Perigonadal Adipocyte Proportions

The cutoff points for small and large adipocytes were defined based on the 25th and 75th percentiles of the CSA from CC group distribution (**Figure 6A**), which were 1,051 µm^2 for small adipocytes and 1,789 µm^2 for large ones.

When the relative percentage of small, medium, and large adipocytes between groups was compared (**Figure 7**), only significant differences in the proportions of small and large of the CC group with those groups that consumed sucrose at some periods of their life were found. In both comparisons, sucrose consumption affected in some way the development of large adipocytes, resulting in a significant decrease in the relative percentage of frequency of small adipocytes (CC: 32.6%, CS: 16.1%, SC: 15.4%, and SS: 17.3%) and an increase in large adipocytes (CC: 29.5%, CS: 54.0%, SC: 52.6%, and SS: 51.0%). The highest percentages in the adipocyte CSA were located between intervals (bin 200) of 1,200–1,800 µm^2 (overall) in all groups (CC: 37.7%, CS: 29.8%, SC: 31.9%, and SS: 32.5%) without differences between them (**Figure 7**).

These differences between the proportion of small and large adipocytes were observed when drawing with different colors the classification of the CSA adipocyte in the photomicrographs taken at ×10, presenting a high proportion of small adipocytes in the CC group (**Figure 6A**) compared to the groups that consumed sucrose at some stage of their life, since the CS (**Figure 6B**), SC (**Figure 6C**), and SS (**Figure 6D**) groups presented a greater number of large than small adipocytes.

The adipocytes of the CC group were observed to be proportionally amorphous, asymmetric, and smaller (**Figure 6A**) compared to those of the CS, SC, and SS groups (**Figures 6B–D**), which were observed to be more rounded, symmetric, and larger.

DISCUSSION

Excessive sugar consumption during gestation is positively associated with maternal weight gain at the end of this period (Renault et al., 2015). Findings of the present study confirm that mothers who consumed sucrose throughout gestation increased the consumption of water with sucrose and in consequence the total carbohydrates amount, resulting in a significant increase in

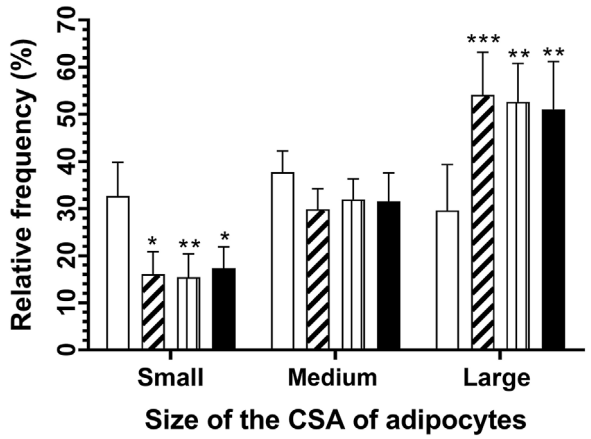

FIGURE 7 | Relative frequency of CSA adipocytes according to their size category for the CC (white bars), CS (diagonal lines bars), SC (vertical lines bars), and SS (black bars) groups. Means ± SEM. Data were analyzed with a two-way ANOVA and Tukey *post hoc*. Significant differences among intervals are shown. *p < 0.05, **p < 0.01, and ***p < 0.001.

the weight at the end of this period. This is because carbohydrate consumption consequently leads to an accumulation of triglycerides in different WAT deposits, which increase their size (hypertrophy) (Morigny et al., 2021). At birth, the consumption of sucrose increased slightly (not significant data) the body length of the offspring. This is likely associated with the glycemic index, which is related to neonatal central adiposity measured by the ratio waist–length in humans (Horan et al., 2014). For its part, birth weight was unaffected by sucrose consumption, as it has been reported in other studies with a moderate maternal nutritional excess (Petherick et al., 2014).

Both in pregnant mothers and adult male groups, there were no differences along the growth trajectory. But a positive relationship was found between the increase in the body weight and weight gain at the end of treatments because the postnatal diet and its interaction with maternal diet, as it had been reported in mice (Mao et al., 2018). Highlighting that in our model, the weight increase begins after the 3rd week of sucrose consumption. This effect on the body weight has been previously described with high carbohydrates diets (Adekunbi et al., 2016; Mao et al., 2018; De León-Ramírez et al., 2021), indicating that the consumption of these sugars (regardless of whether it is mono or disaccharide) is associated with the regulation of solid food intake to adapt energy provided by the increase in the consumption of sugar water (Sheludiakova et al., 2012; Cervantes-Rodríguez et al., 2014).

Respect to the protein consumption, despite the fact that their caloric intake was reduced, it did not imply a deficiency in the consumption of this nutrient, since both in pregnant females that consumed sucrose (S, 18.5%), as in the adult offspring that postnatally consumed it (CS, 19.4%; SS, 19.2%) and in those males control that came from mothers that consumed during pregnancy and breastfeeding (SC, 23%). This intake ranged from 15% to 20% of total caloric intake, which is considered within the optimal range, even between 20% and 25%, which is desirable for these animals (Moro et al., 2021).

We did not observe changes in the number of seminiferous tubules counted, since, as already described, the rat testicle is composed of approximately 20 seminiferous tubules (Clermont and Huckins, 1961), with a spatial distribution that depends on its connection with the rete testis (Nakata et al., 2015). Also, because the complete development of the number of these tubules occurs during the gestational stage, changes in this variable have been reported when interventions by mutagenic, teratogenic, or carcinogenic agents have been carried out at this stage (Tirpák et al., 2021).

Blood–testicular barrier (HTB) maintains the balance in the testicular environment, promoting a healthy and complete development of cell types in these organs (Banks et al., 1999). However, it has been reported that high concentrations of sugar ingested in postnatal stages (Mao et al., 2018; De León-Ramírez et al., 2021) can modify apoptotic processes in the germ and Sertoli cells (Mao et al., 2018), as well as the CSA, epithelial area (germinative, luminal, interstitial), and testicular weight (De León-Ramírez et al., 2021). Probably due to significant increases in the leptin levels that could cross HTB and modify the communication with testicular triglycerides or with important hormones such as testosterone (Mao et al., 2018; De León-Ramírez et al., 2021). In the same context, Despite not finding structural changes in the morphology of the main cell types intra (Sertoli cells, spermatogonia, spermatocytes, spermatids, and spermatozoa) and extra tubular (Leydig cells), we observed a high proportion of macrophages and interstitial mast cells in groups that consumed sucrose maternal and postnatally, which can be related to a pro-inflammatory status with the possibility of affecting testosterone synthesis and its consequent metabolic and cellular interactions (Harris et al., 2016; Ye et al., 2021), including its communication with WAT deposits like the perigonadal.

WAT had hypertrophy and hyperplasia, and the balance between these two mechanisms is an important factor determining the ultimate outcome in lipid storage homeostasis. For this reason, the determination of the size and number of adipocytes in different WAT deposits has focused on finding new and better strategies for their evaluation and understanding of the involved metabolic dynamics (Ibáñez et al., 2018).

Although in our study a Gaussian-type distribution was determined for adipocytes size, it also present characteristics that could be considered a gamma distribution, which has been used to evaluate the size adipocytes distribution from offspring due to the effect of obesogenic maternal diet (Ibáñez et al., 2018).

As it has been reported for the WAT, an increase in the content of triglycerides may be associated with hypertrophy and/or hyperplasia of adipocytes of the PAT (Morigny et al., 2021). We found that both processes were triggered and increased by the high postweaning sucrose intake and programmed by the same diet during pregnancy and lactation. A decrease in the relative percentage of small adipocytes was observed in adulthood, leading to a significant increase in the number and the

percentage of large adipocytes in the SC and SS groups. Although maternal diet promotes changes in the WAT physiology, metabolism, and size in adult offspring (Cervantes-Rodríguez et al., 2014; Ibáñez et al., 2018), our data support the theory of fetal programming (Barker, 1990) in periods of development, such as pregnancy and breastfeeding (Renault et al., 2015; Ibáñez et al., 2018; Corona-Quintanilla et al., Online ahead of print), which is probably due to the WAT ontogenesis approximately begins at embryonic day 13.5 (Berry et al., 2016) from the dermomyotome of the mesoderm, and the origin of hypertrophic adipocytes has been speculated since the fetal period and continues after birth (Nielsen et al., 2016; Sebo and Rodeheffer, 2019).

Despite weight gain in PAT and probable metabolic dysfunctions (increased triglycerides storage and adipogenesis) in adipocytes during pregnancy and breastfeeding have been reported, which can lead to the development of hypertrophy and hyperplasia in adulthood, the consumption of a high-sugar diet from early childhood to adulthood promotes the development of these pathologies in adulthood. Although this programming was due to the high consumption of carbohydrates in the maternal diet, the chronic consumption during postnatal life (second hit) of additional simple carbohydrates (such as sucrose) can increase the weight gain in the WAT of the perigonadal deposit and changes the size distribution of its adipocytes. This has been reported in the retroperitoneal adipose tissue of male offspring of rats with maternal obesity on high-fat diets (Ibáñez et al., 2018) and in models with maternal protein deficiency and sucrose consumption (second hit) in postnatal life (Cervantes-Rodríguez et al., 2014).

Conventional approaches often consider variation in the distribution of small and large adipocytes as outliers, rather than functionally significant data with important physiological implications (Ibáñez et al., 2018).

The congruence between results of our work and those of other colleagues (Cervantes-Rodríguez et al., 2014; Ibáñez et al., 2018) could explain the metabolic heterogeneity between obese and non-obese individuals (McLaughlin et al., 2014; Ibáñez et al., 2018), since none of our individuals showed an increase in the body weight considered within the range of obesity, but there is an increase in the body weight of the TAP and in the proportion and size of large adipocytes in this WAT deposit, which is the result of a significant increase in the accumulation of lipids in mature adipocytes (Cervantes-Rodríguez et al., 2014, Cinti et al., 2019), leptin increase (Cervantes-Rodríguez et al., 2014; Ibáñez et al., 2018), and changes in adipogenesis process (Cinti et al., 2019). Further studies are needed to elucidate the development of changes in this process. This together provides a better understanding of the metabolic implications of the variation in WAT.

In addition, hypertrophy of adipocytes from different fatty deposits, including the perigonadal is associated with an increase in the leptin expression, leptin mRNA levels, presence of macrophages, and high levels of IL-6 and TNFα (McLaughlin et al., 2014), as well as a decrease in the insulin levels (Guo et al., 2004).

This increase in the leptin levels (associated with perigonadal adipocyte hypertrophy) can decrease testicular testosterone synthesis (Tena-Sempere et al., 1999; Rato et al., 2013), since there are leptin receptors in Leydig cells, and it has been proven that this protein can cross the HTB and binds to its receptor (Banks et al., 1999), negatively affecting different processes, such as spermatogenesis (germ cell apoptosis), Sertoli cell metabolism (Banks et al., 1999; Mao et al., 2018; Sengupta et al., 2019), and steroidogenesis (by feedback *via* the hypothalamus and pituitary gland), also due to a decrease in insulin associated with high levels of leptin after high sugar diets (Rato et al., 2013; Sengupta et al., 2019). Furthermore, at the level of sperm maturation in the epididymis, it subsequently decreases sperm quality (De León-Ramírez et al., 2021), and even neuroendocrine ones, such as the displacement of adequate sexual behavior (Shulman and Spritzer, 2014). However, more studies are needed to corroborate these hypotheses.

This decrease in the synthesis of testosterone can be accentuated by the pro-inflammatory environment in the interstitial space of the testis, characterized by an increase in the presence of macrophages (Harris et al., 2016), which could be observed in our results.

During the excessive consumption of nutrients, the expansion capacity of WAT reaches its limit and there is a strong association between the size and death of adipocytes (Strissel et al., 2007; Morigny et al., 2021). This triggers changes in the immune cells surrounding hypertrophic WAT adipocytes, increasing the synthesis of pro-inflammatory macrophages and their production of cytokines such as IL-6, TNFα, and oncostatin M (Crewe et al., 2017; Cinti et al., 2019; Morigny et al., 2021). This chronically leads to leptin increase and impaired insulin signaling in adipocytes, increased inflammation, and prolonged worsening of adipose tissue dysfunction (Crewe et al., 2017; Cinti et al., 2019). Although adipose tissue inflammation has detrimental effects, it is possible adaptive and homeostatic roles for pro-inflammatory signaling in WAT expansion and function (Cinti et al., 2019). Thus, the associated metabolic damage (insulin resistance, rise in lipid accumulation, synthesis and expression of leptin, and pro-inflammatory status) (Cervantes-Rodríguez et al., 2014; Ibáñez et al., 2018) can be significantly increased, resulting in alterations in the size of the adipocytes and a considerable increase in the weight of the PAT. Also, it can affect the testosterone synthesis in the testis, promoting actions of leptin in Leydig cells (Banks et al., 1999; Guo et al., 2004).

The results of this study show that the high consumption of simple carbohydrates such as sucrose during pregnancy promotes the establishment of hyperplasia and hypertrophy in WAT deposits such as perigonadal in the male offspring. The development of these processes significantly depends on the amount and type of carbohydrates ingested or not throughout postnatal life. This invites us to pay special attention to the unnecessary consumption of simple carbohydrates during the

stages of pregnancy, lactation, early childhood, adolescence, and adulthood.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Comité de Ética del Centro Tlaxcala de Biología de la Conducta del Universidad Autónoma de Tlaxcala.

AUTHOR CONTRIBUTIONS

R-AJ initiated the research work, conceived the study, and designed the experiment. C-SG analyzed, edited, interpreted, and redacted the data and wrote the manuscript. C-RM analyzed the data, contributed analysis tools, provided valuable suggestions during the experiments, and the writing of the manuscript. R-AJ, N-TL, C-RE, and A-CL edited the manuscript and provided valuable suggestions during the experiment. X-AN analyzed the imaging results and provided valuable suggestions during the writing of the manuscript. ZE provided valuable suggestions during the writing of the manuscript, specifically when analyzing the results for the writing of the discussion. N-TL and C-RE contributed reagents and materials. All authors contributed to the article and approved the submitted version.

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Fear Stress During Pregnancy Affects Placental m6A-Modifying Enzyme Expression and Epigenetic Modification Levels

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As the hub connecting mother and offspring, the placenta's normal development is vital for fetal growth. Fear stress can cause some structural alterations in the placenta and affect placental development and function. N6-methyladenosine (m6A) is the most common mRNA modification and is involved in regulating the development of the placenta and embryo. There are no reports on the potential role of m6A modification in placental damage caused by fear stress during pregnancy. In this study, we demonstrated that fear stress during pregnancy increases the levels of methylated enzymes (METTL3, METTL14, and WTAP), decreases the levels of demethylase FTO, and increases the overall methylation levels in the placenta of pregnant rats. MeRIP-seq data analysis revealed 22,010 m6A peaks associated with 12,219 genes in the placenta of the model and 21,060 m6A peaks associated with 11,730 genes in the placenta of the control. The peaks were mainly concentrated in the coding region and the 3′ untranslated region. In addition, 50 genes with abnormal modification and expression (double aberrant genes) were screened out by combining MeRIP-seq and RNA-seq data. *Mefv*, *Erbb2*, and *Cgas* were selected from 50 double aberrant genes, and MeRIP-qPCR and real-time quantitative polymerase chain reaction were used to verify their modification and expression levels. Our findings suggest that m6A modifications play an important role in placental dysfunction induced by fear stress during pregnancy.

Keywords: fear stress, placenta, N6-methyladenosine, methylation enzymes, demethylase, double aberrant genes

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INTRODUCTION

Increasing stress and trauma due to factors such as competition and emergencies have led to a significant increase in psychologically-induced illnesses (Turner et al., 2020). Pregnant women are especially susceptible to psychological problems caused by external stimuli during pregnancy (Smith et al., 2020). Fear stress causes significant physical and mental harm to pregnant women and dramatically increases the risk of fetogenic diseases and fetal malformations (Tsui et al., 2006; Glover and Capron, 2017; Mizrak Sahin and Kabakci, 2021). Therefore, the prevention and treatment of fetal diseases have become an urgent social issue.

The hypothalamic–pituitary–adrenal (HPA) axis has been the focus of most studies on the relationship between stress and fetal stunting. It is hypothesized that pregnancy stress can overactivate maternal HPA axis function, thereby resulting in increased glucocorticoid (GC) levels (Howland et al., 2017; Anifantaki et al., 2021; Rybnikova and Nalivaeva, 2021). Maternal

GC overexpression causes alterations in intrauterine HPA axis programming in the offspring, thereby affecting the development and health of the offspring (Howland et al., 2017). However, it has also been suggested that the placenta is a promising programming vehicle (Montoya-Williams et al., 2018; Musillo et al., 2022). Recent studies have found that the genetic changes regulated by placental N6-methyladenosine (m6A) modifications play a pivotal role in fetal growth and preeclampsia (PE) development (Wang et al., 2020a; Wang et al., 2021).

M6A is the most prevalent, abundant, and highly conserved internal modification of eukaryotic mRNA. It is dynamically regulated by "writers" and "erasers," influencing the destiny of transcripts through "readers." (He et al., 2019; Zaccara et al., 2019). Most of the m6A methylation on the mRNA is installed by a writer complex consisting of the core subunits METTL3 and METTL14, and additional adaptor proteins, including WTAP, Virma, ZC3H13, Hakai, and RBM15/15B. Another writer, METTL16, can install m6A in the sequence of UAC (m6A) GAGAA at the top of the hairpin structure in the transcript MAT2A. Two erasers, FTO and ALKBH5, have been identified for m6A demethylation on mRNA. Reader proteins prefer binding to m6A-containing RNAs using different mechanisms. Proteins containing the YTH domain (YTHDF1-3, YTHDC1-2) directly recognize m6A methylation using a well-characterized YTH domain. RNA-binding proteins such as IGF2BP1-3 and FMR1 prefer m6A-containing RNAs through tandem common RNA-binding domains (RBDS) (Frye et al., 2018; Shi et al., 2019). One study revealed that fear stress could regulate the expression of m6A effectors in different regions of the brain, thereby affecting m6A modification levels (Widagdo et al., 2016; Walters et al., 2017; Engel et al., 2018). However, it is unknown whether fear stress triggers placental dysfunction by altering the levels of placental m6A effectors and m6A modification.

Herein, we studied the changes in methylation enzymes (METTL3, METTL14, and WTAP) and demethylases (FTO and ALKBH5) in the placenta caused by fear stress during pregnancy as well as m6A modification levels of total RNA in the placenta. All differentially modified and aberrantly expressed genes were screened using RNA-seq and MeRIP-seq. Accordingly, it was hypothesized that an imbalance in placental m6A modifications is the causal mechanism underlying placental damage induced by fear stress.

MATERIALS AND METHODS

Animals

Wistar rats (n = 55; 35 females and 20 males; weight 260 ± 30 g) were purchased at 11 weeks of age from the Beijing Vital River Laboratory Animal Technology Co., Ltd. [License No.: SCXK (Beijing) 2016-0006; Animal Certificate No.: 11400700319215]. The rats were fed at the Experimental Animal Center of the Henan University of Chinese Medicine (Henan, China) and housed in sterile animal colonies under the following conditions: 25 ± 3°C temperature, 45 ± 5% humidity, and 12 h light/dark cycle. This study was conducted at Henan University of Chinese Medicine according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The procedures were approved by the Animal Ethics Committee of Henan University of Chinese Medicine (permit number: DWLL2018030017).

Pregnant Rat Model of Fear Stress

After 1 week of adaptive feeding, all the rats were subjected to a baseline test. First, female rats with consistent scores were selected. Next, 15 male rats with the consistent baseline scores were selected as mating rats, and the rest were used as electroshock rats. Finally, according to the 2:1 mating cage ratio, 24 pregnant female rats were randomly divided into control group and fear stress model group, with 12 rats in each group.

The fear stress model was established according to modified bystander electroshock method (Geng et al., 2019). In this method, a stimulus was applied once per day for 20 days. The electroshocked and model rats were placed in corresponding chambers of a homemade electroshock communication box at the same time every day. The electroshocked rats received electric shocks in the chamber, whereas the model rats received fear messages from the electroshocked rats via sight, sound, and smell stimuli from their position in the spectator chamber, thereby generating fear and corresponding psychological stress.

Behavioral Tests

Open Field Test

The open field test was used to evaluate exploratory behavior, anxiety, and depression in animals. The rats were placed in an open black box (dimensions, 100 × 100 × 40 cm^3). The bottom of the box was divided into 25 squares of equal size by white lines, and a quiet experimental environment was ensured. Each rat was lifted by grasping the tip of its tail at one-third of the root and was gently placed in the middle of the open field chamber. The number of times it crossed the grid (we scored one point for crossing one gridline with both hind limbs) or stood upright (we scored one point for lifting both forelimbs off the ground) within a 3-min period following 1 min of adaptation was recorded. Immediately after the rat was subjected to the test, we wiped the inner walls and bottom of the box with alcohol and ventilated the chamber for 2 min before performing the test on the next rat.

Fear Condition Test

The fear condition test was performed to evaluate the acquisition, extraction, and regression of fear memory in animals. On the day before the test, the rats were acclimatized by placing them in a scenario fear box for 2 min, followed by a stimulation procedure. This procedure consisted of 30 s of noise stimulation (1 Khz, 80 Db) interspersed with 2 s of light stimulation (310 Lux) and 2 s of electrical stimulation (0.5 mA), followed by 30 s of rest. Acclimatization comprised four cycles of this stimulation procedure, which lasted 6 min. The fear condition test was conducted 24 h later. The test time was 6 min, including 2 min of adaptation and 2 min of noise and light stimulation of the same intensity but without electrical stimulation. A computer automatically tracked the duration of rigidity and immobility in the rats as "freezing time," which was used as an indicator to assess fear in the stress model rats.

Placenta Collection

After the behavioral test, the animals were anesthetized using 2% pentobarbital (3 ml/kg). Blood was drawn from the abdominal aorta, and 24 pregnant rats were sacrificed by cervical dislocation. All the placentas were manually separated from the endometrium. The weight and diameter of the placentas in each litter were evaluated, and two placentas from each litter were randomly selected. The round tissue with a diameter of 5 mm around the umbilical cord of each placenta was taken out, and stored in a cryogenic refrigerator at −80°C for subsequent RNA and protein extraction. The remaining placenta was used for another experiment. All the above steps were completed in an aseptic environment, and the sample collection process was completed within 5 min of separating the placenta *in vitro*.

Enzyme-Linked Immunosorbent Assay

The levels of ACTH, GC, and estriol in the serum of pregnant rats were determined using enzyme-linked immunosorbent assay (n = 12). All steps were conducted according to the manufacturer's instructions: 1) the absorbance of each sample was measured at 450 nm, 2) the absorbance served as the vertical coordinate and a corresponding standard concentration served as a horizontal coordinate to plot a standard curve, and 3) the ACTH, GC, and estriol levels in each sample were calculated according to the regression equation of the standard curve.

Histological Analyses of the Placentas

The placentas were fixed in 4% paraformaldehyde solution, dehydrated with a gradient solution of ethanol and water, and purified by xylene. Subsequently, they were paraffin-embedded and cut into 5-μm thick sections. The sections were stained with hematoxylin and eosin. Finally, micrographs were obtained using a light microscope (Nikon, E100, Japan).

Western Blot Analysis

Six biological replicates were selected from each group, and RNA and protein were extracted for western blot analysis and real-time quantitative polymerase chain reaction (RT-qPCR). RIPA lysate (Beyotime Biotechnology, Shanghai, China) was used to extract total protein from the placental tissues. The total protein concentration was determined according to the instructions of the BCA kit. The total protein samples were separated via SDS-PAGE (stacking gel, 4%; separating gel, 8%). After electrophoresis, the separated protein bands were transferred to PVDF membranes. Subsequently, the membranes were blocked with a blocking solution containing 5% skimmed milk powder and TBST solution for 1 h. The membranes were then incubated overnight at 4°C with a primary antibody, e.g., anti-METTL3 or anti-GAPDH (1:1,000; Abcam, Cambridge, United Kingdom); anti-METTL14, anti-WTAP, or anti-FTO (1:1,000; CST, Boston, MA, United States); or anti-ALKBH5 (1:1,000; Novus Biologicals, Littleton, CO, United States). The membranes were then washed thrice for 10 min each with TBST. A secondary antibody [goat anti-rabbit IgG H&L (HRP), 1:10,000; rabbit anti-mouse IgG H&L (HRP), 1:10,000; Abcam, Cambridge, United Kingdom] was then added, and the membrane was incubated for 1 h. The membrane was subsequently washed thrice with TBST for 10 min. The blots were visualized using a Tanon 6,600 luminescence imaging workstation (Tanon, Shanghai, China) and optical density values were analyzed using Image Pro Plus 6.0 software. The protein levels were measured and expressed relative to the expression level of the internal reference protein GAPDH.

TABLE 1 | Primers used for real-time quantitative polymerase chain reaction.

Primers	Sequence (5′→3′)	
FTO	Forward	5′-GTGTGACAAATGCCGTGCTT-3′
	Reverse	5′-TGCTGTGCTGGTAGAGTTCG-3′
ALKBH5	Forward	5′-ACGGCCTCAGGACATCAAAG-3′
	Reverse	5′-AAGCATAGCTGGGTGGCAAT-3′
METTL3	Forward	5′-ATGTGCAGCCCAACTGGATT-3′
	Reverse	5′-CTGTGCTTAAACCGGGCAAC-3′
METTL14	Forward	5′-CACCGCTACCAGTCTTGGAC-3′
	Reverse	5′-ATCTGCACTCTCAGCTCCCA-3′
WTAP	Forward	5′-CCTCGCCTCGTCTCTTCTGG-3′
	Reverse	5′-GTCATCTTGTACCCCGAGACG-3′
Mefv	Forward	5′-AGAAATGCTGGGCTCCGAAAT-3′
	Reverse	5′-GACGGATGAAGGTAATCTTGAGG-3′
Erbb2	Forward	5′-TGGAGGAGTGCCGAGTATGGA-3′
	Reverse	5′-AATGATGAATGTCACCGGGCT-3′
Cgas	Forward	5′-AGCCAGACAAGCTAAAGAAGGTG-3′
	Reverse	5′-GCAGCAGTTGATCCACGACTTTAT-3′
GAPDH	Forward	5′-TGATTCTACCCACGGCAAGTT-3′
	Reverse	5′-TGATGGGTTTCCCATTGATGA-3′

RT-qPCR

Total RNA was extracted from the placental tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, United States). cDNA was extracted using the SoFast EvaGreen Supermix (Bio-Rad Laboratories, CA, United States), and RT-qPCR was performed using an ABI 7500 real-time fluorescence quantitative PCR instrument (ABI, CA, United States). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, which were then normalized against GAPDH mRNA levels. The specific primer sequences are shown in **Table 1**.

m6A RNA Methylation Assay

Total RNA was extracted from each group of six samples. The EpiQuik m6A RNA Methylation Quantification Kit (Epigentek, United States) was used to assess the global m6A modification levels of the mRNA. Briefly, 200 ng of poly(A) purified RNA was added to each well, following which the relevant antibody was added to each well individually at the appropriate dilution. The OD450 of each well was measured. m6A modification levels were quantified from the standard curve and calculated.

MeRIP-Seq and RNA-Seq

Sequencing was performed for three rats from each group. Poly(A) RNA was purified from 50 μg of total RNA using Dynabead Oligo (dT) 25-61005 (Thermo Fisher, CA, United States) in two purification rounds. Poly(A) RNA was then fragmented into

TABLE 2 | Sequences of primers used for m6A-qPCR.

Primers	Sequence (5′→3′)	
Mefv	Forward	5′-AGGAATGGAATGAATAGGAA-3′
	Reverse	5′-TGGACCCCAGTCAGAGTAAC-3′
Erbb2	Forward	5′-ACCCTGAATACTTAGTACCGAG-3′
	Reverse	5′-GAGTTCTGGTCCCAGTAATAGAG-3′
Cgas	Forward	5′-GATGTTCATGTAACCCTGGCT-3′
	Reverse	5′-TGCTATCAAGCATGGTAGCAC-3′

small pieces using a Magnesium RNA Fragmentation Module (NEB, cat.e6150, United States) at 86°C for 7 min. A portion of the fragmented RNA was used as an RNA-seq library. Cleaved RNA fragments were then incubated for 2 h at 4°C with an m6A-specific antibody (No. 202003, Synaptic Systems, Germany) in IP buffer (50 mM Tris-HCl, 750 mM NaCl, and 0.5% Igepal CA-630). The IP RNA was reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen, cat. 1896649, United States). The cDNA was then used to synthesize U-labeled second-stranded DNA using *Escherichia coli* DNA polymerase I (NEB, cat.m0209), RNase H (NEB, cat.m0297), and dUTP solution (Thermo Fisher, cat.R0133). An A base was then added to the blunt ends of each strand to prepare them for ligation to the indexed adapters. Each adapter contained a T-base overhang for ligating the adapter to the A-tailed fragmented DNA. Single- or dual-index adapters were ligated to the fragments, and size selection was performed using AMPureXP beads. The U-labeled second-stranded DNA was treated with heat-labile UDG enzyme (NEB, cat.m0280), and the ligated products were amplified using PCR at the following conditions: initial denaturation at 95°C for 3 min; eight cycles of denaturation at 98°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s; and final extension at 72°C for 5 min. The average insert size of the final cDNA library was 300 ± 50 bp. Finally, we performed 2 × 150 bp-paired-end sequencing (PE150) on Illumina Novaseq™ 6,000 (LC-Bio Technology Co., Ltd., Hangzhou, China) following the manufacturer's recommended protocol.

Gene-Specific m6A qPCR

The IP products were collected and reverse transcribed into cDNA according to the MeRIP-seq process described above. Gene-specific primers were then designed for qPCR-based quantification. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and were normalized against GAPDH mRNA expression levels. The primers used for PCR are presented in **Table 2**.

Bioinformatics Analysis of MeRIP-Seq and RNA-Seq Data

We performed quality control of the raw data of placental samples using fastp (v0.19.4). We used the sequence alignment tool HISAT2 (v2.0.4) to compare the genome (v101) to reference sequences. Peak calling and gene difference peak analysis were performed using the exomePeak (v1.9.1) package for R (version 4.1.2). All peaks were annotated using ChIPseeker (v1.18.0). Finally, we performed motif analysis using MEME2 (v4.12.0) and HOMER (v4.1). Differential peaks and differentially expressed genes in the placental samples were identified if the sample showed a fold count of ≥1.5 and a p-value of <0.05. Gene enrichment analysis was performed based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations. The overall experimental flow is shown in **Figure 1**.

Statistical Analyses

All summary data were expressed as mean ± standard deviation, and all statistical tests were performed using SPSS version 21.0 (IBM SPSS, United States). Comparisons between two groups were performed using *Student's t-test*, and comparisons of non-normally distributed data were performed using *Mann–Whitney U test*. For all tests, a p-value of <0.05 indicated statistical significance.

RESULTS

Evaluation of the Pregnant Rat Models of Fear Stress

Open field and fear condition tests were used to assess emotional changes in the pregnant rats. Compared with the control rats, horizontal and vertical scores were lower and freezing time was longer in the model rats (**Figures 2A,B**). In addition, there was a significant increase in ACTH and GC levels in the model rats (**Figure 2C**). These results indicate that the fear stress model was successfully established and could be used for further research.

Reduced Placental Weight and Impaired Placental Function Due to Fear Stress

After fear stimulation, we observed that the live births of model rats had decreased by 28%; individual placenta weights, 20%; and total placenta and offspring weights, 19%. (**Figures 2D–F**). These findings indicate that fear stress can decrease placenta weight and offspring viability. We observed that serum estriol levels in the models were markedly reduced (**Figure 2G**), suggesting that fear stress impairs placental function.

Histological Analysis of the Placenta of the Pregnant Rat Models of Fear Stress

Placental development was analyzed from the perspectives of gross morphology and histology. The placenta was smaller and the weight of the offsprings was lower in the model rats than in the control rats (**Figure 2H**). Histological analysis revealed that the placenta was tightly arranged with abundant vascularity and well-developed branching at the interface of the labyrinth layer in the control rats, whereas it showed extensive disorganization of the tissue structure, narrowed capillaries, and multiple vacuoles of varying sizes in the model rats (**Figure 2I**).

Fear Stress Alters Placental m6A-Modifying Enzyme Expression

To examine the placental expression of methylesterase, we randomly selected six placental tissue from each group. Our

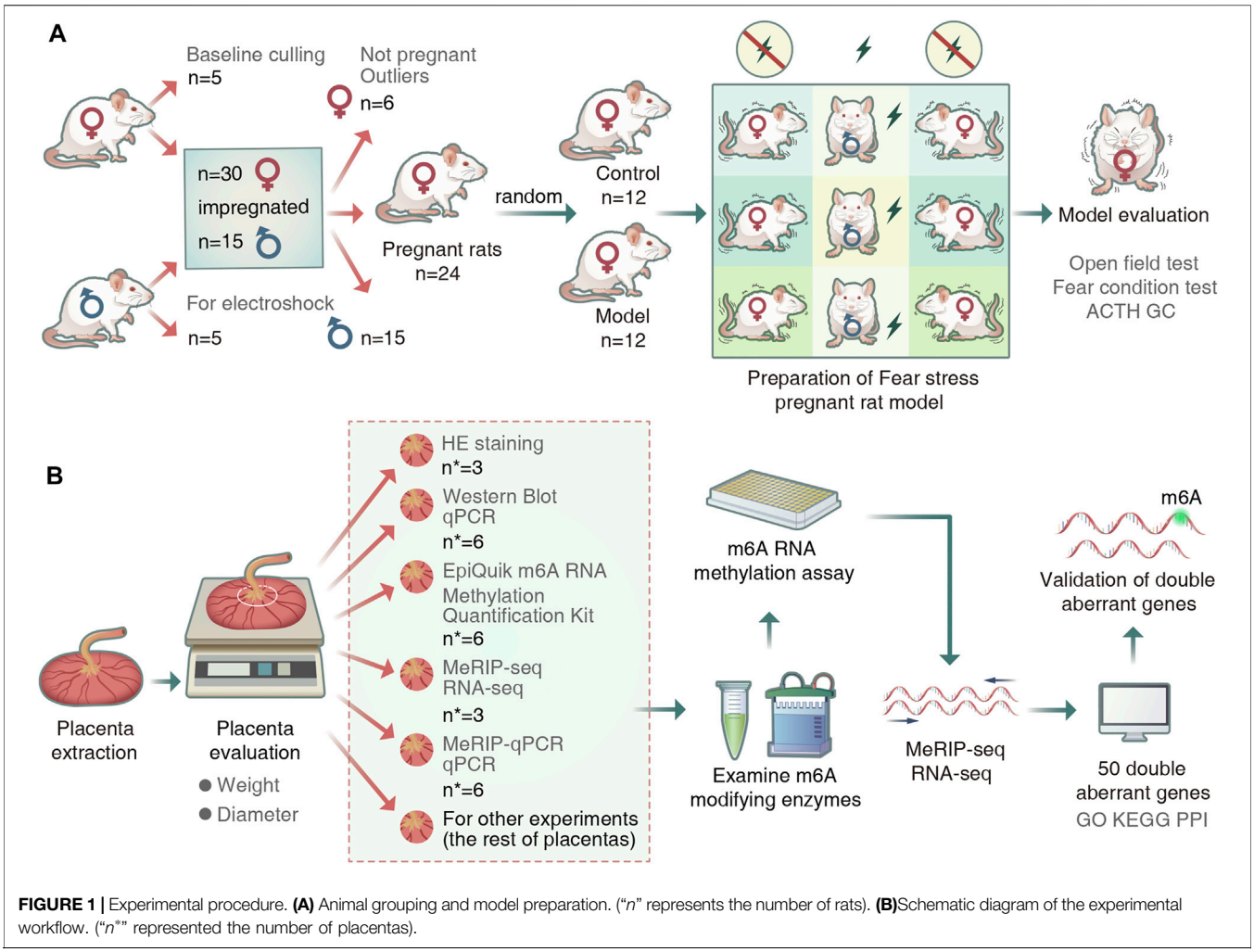

FIGURE 1 | Experimental procedure. **(A)** Animal grouping and model preparation. ("*n*" represents the number of rats). **(B)**Schematic diagram of the experimental workflow. ("*n**" represented the number of placentas).

results showed that the mRNA expression levels of METTL3, METTL14, and WTAP were significantly upregulated; those of FTO were significantly downregulated; and those of ALKBH5 did not change significantly in the model rats (**Figure 3A**). The protein expression levels of METTL3, METTL14, WTAP, FTO, and ALKBH5 were consistent with their mRNA expression levels (**Figure 3B**). Freezing time is considered an effective indicator for evaluating animal fear. We assessed the correlation between the expression levels of the four enzymes and freezing time and found that FTO was negatively correlated with freezing time ($r = -0.82$, $p < 0.05$). In addition, FTO was positively correlated with total fetal weight and single placental weight (**Figure 3C**; $r = 0.85$ and $r = 0.83$, respectively; $p < 0.05$). Therefore, we speculated that FTO plays a major role as a core enzyme in placental m6A modification imbalance induced by fear stress.

m6A Methylation Map in Placental Tissue Samples From Model and Control Rats

Six placental tissue were randomly selected from each group to assess the overall level of m6A modification in the placenta. The levels of m6A modification in the model rats were significantly higher than those in the control rats (**Figure 4A**), suggesting that m6A modification is involved in placental damage caused by fear stress. Next, we selected three placental tissue per group for m6A-seq, and after pre-processing and quality control of the raw data, approximately 45 million valid data were obtained (**Table 3**). Of these data, 22,010 m6A peaks were associated with 12,219 gene transcripts in the model group, whereas 21,060 m6A peaks were associated with 11,730 gene transcripts in the control group. Further statistical analysis of the transcripts containing m6A modifications in both groups showed that the majority of the modified transcripts contained 1–3 m6A peaks (**Figure 4B**). To clarify the observed distribution of m6A sites on the transcriptome, m6A peak datasets were divided based on gene location. Specifically, m6A sites are mainly present in five regions: 5′ untranslated region (UTR), start codon, coding DNA sequence (CDS), stop codon, and 3′ UTR. We found that the m6A peaks were mainly located at the beginning of the CDS and 3′ UTR. This is consistent with the distribution of peaks previously found in mammalian systems. Interestingly, a unique m6A peak was found at the end of the 5′ UTR in our study (**Figure 4C**).

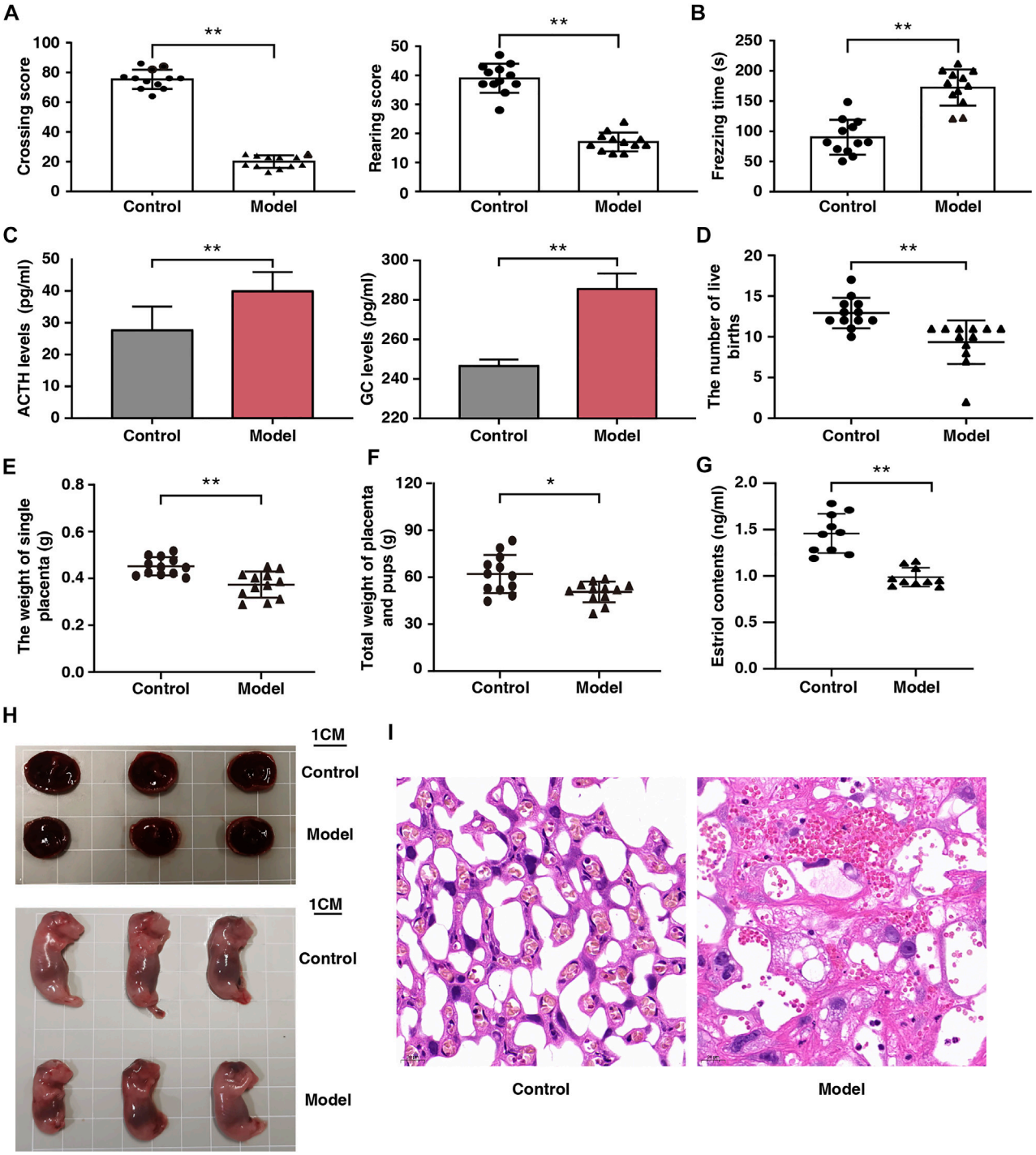

FIGURE 2 | Effect of fear stress during pregnancy on the behavior and fetal outcomes of pregnant rats. **(A)** Crossing and rearing scores of pregnant rats in control and model groups ($n = 12$, $^{**}p < 0.01$, $^{**}p < 0.01$). **(B)** Freezing time of pregnant rats in control and model groups ($n = 12$, $^{**}p < 0.01$). **(C)** ACTH and GC levels in control and model groups ($n = 12$, $^{**}p < 0.01$, $^{**}p < 0.01$). **(D)** The number of live birth of pregnant rats in control and model groups ($n = 12$, $^{**}p < 0.01$). **(E)** The weight of single placenta of pregnant rats in model and control groups ($n = 12$, $^{**}p < 0.01$). **(F)** Total weights of the placenta and pups of pregnant rats in control and model groups ($n = 12$, $^{*}p < 0.05$). **(G)** The estriol levels of pregnant rats in control and model groups ($n = 12$, $^{**}p < 0.01$). **(H)** Placenta and morphological results of offspring in control and model groups (Scale bars, 1 cm). **(I)** Placental sections obtained from pregnant rats (stained with hematoxylin and eosin; scale bar, 20 μm). ACTH, Adrenocorticotropic hormone; GC, Glucocorticoid.

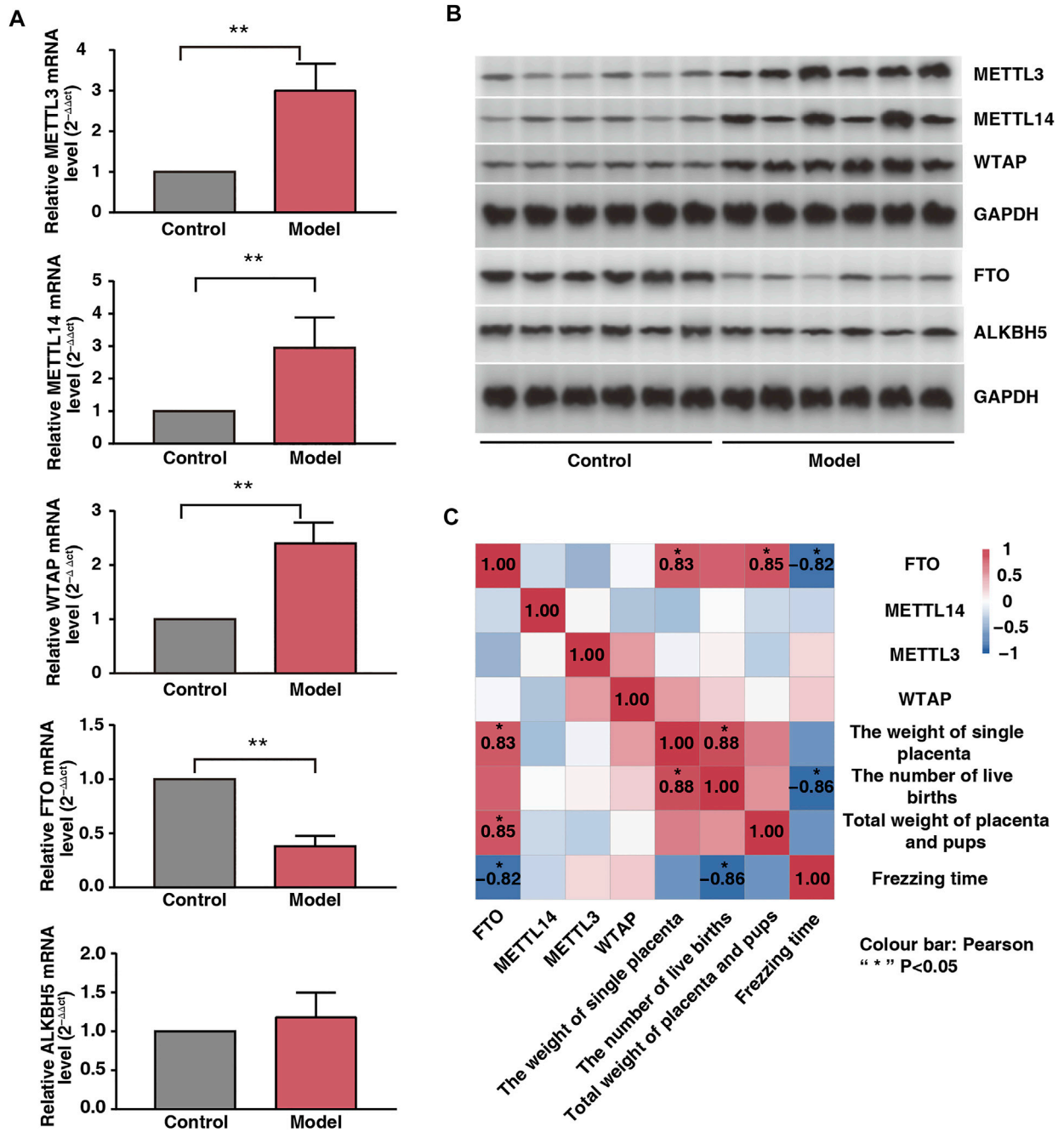

FIGURE 3 | Effect of fear stress during pregnancy on placental methylase. **(A)** PCR analysis of enzymes (METTL3, METTL14, WTAP, FTO and ALKB5) in control and model groups ($n^* = 6$, $^{**}p < 0.01$, $^{**}p < 0.01$, $^{**}p < 0.01$, $^{**}p < 0.01$, $p > 0.05$). **(B)** Western blot analysis of enzymes (METTL3, METTL14, WTAP, FTO and ALKB5) in control and model groups ($n^* = 6$, $^{**}p < 0.01$, $^{**}p < 0.01$, $^{**}p < 0.01$, $^{**}p < 0.01$, $p > 0.05$). **(C)** Correlation analysis of four enzymes (METTL3, METTL14, WTAP, and FTO) with the emotions and fetal outcomes of pregnant rats.

To determine whether the m6A peaks contained the classical m6A sequence RRACH (where R represents a purine, A represents m6A, and H represents a nonguanine base), we performed *de novo* motif analysis on the detected m6A sites and found a classical motif, the GGACU sequence, in both groups (**Figure 4D**). This finding strengthened the possibility of an m6A peak being present.

GO and KEGG Analysis of Differentially Methylated mRNAs

To further analyze the abundance of m6A peaks in the two groups, we used exomePeak to screen 784 differential peaks at a fold change of ≥1.5 and a *p*-value of <0.05, including 470 hypermethylated sites and 314 hypomethylated sites (**Figure 4E**). The number of

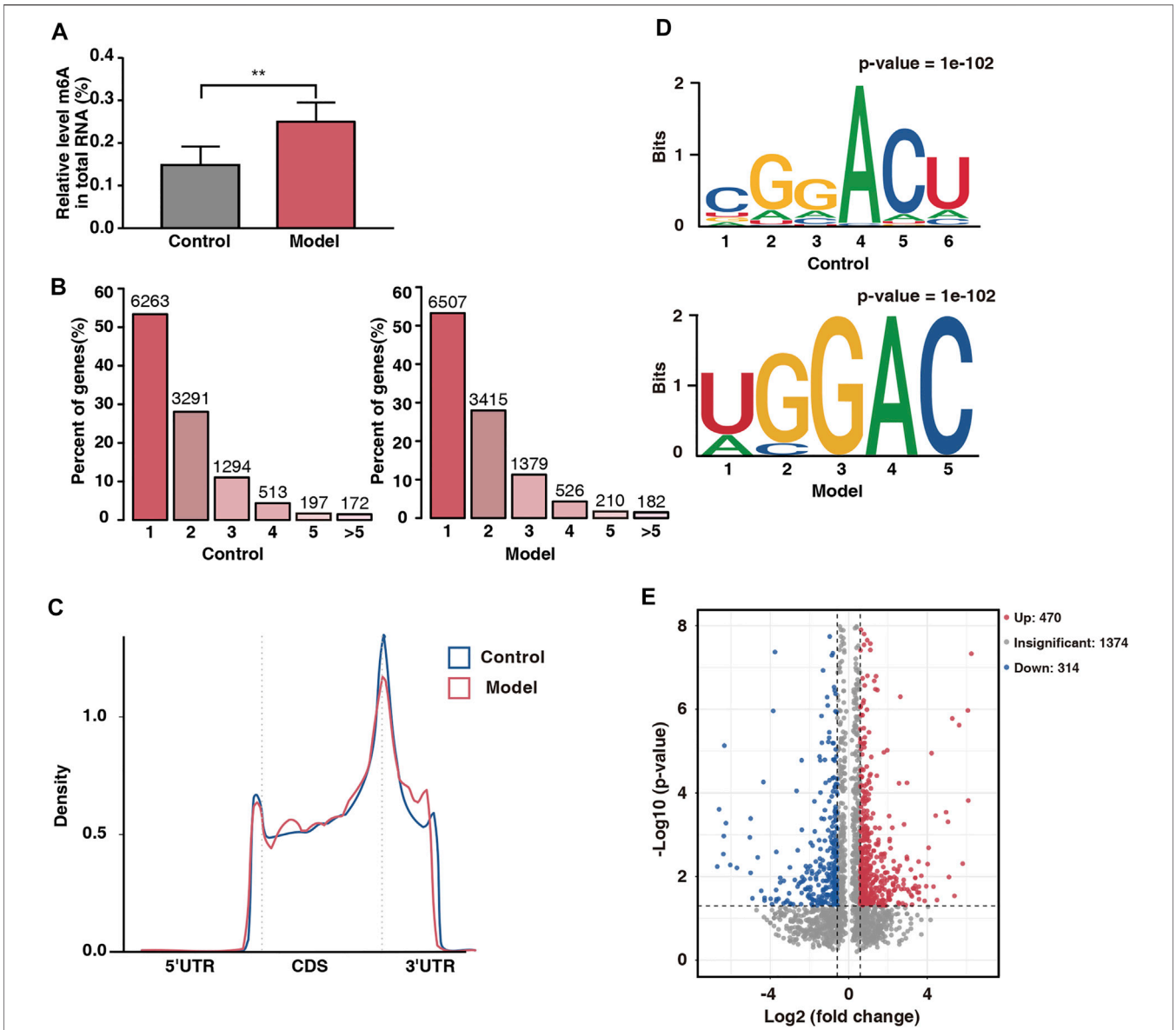

FIGURE 4 | Overview of placental m6A methylation in fear stress during pregnancy. **(A)** m6A modification levels of total RNA in control and model groups ($n^* = 6$, $^{**}p < 0.01$). **(B)** Number of peaks of m6A-modified genes in control and model groups. Sequence motifs with m6A peak regions in control and model groups. **(C)** Density of differential m6A peaks along transcripts. **(D)** Sequence motifs with m6A peak regions in control and model groups. **(E)** Distribution of differentially modified m6A peaks in control and model groups (fold change ≥1.5 and $p < 0.05$).

hypermethylated sites was significantly greater than that of hypomethylated sites, suggesting that fear stress increases placental m6A modification levels.

To explore the function of m6A-modified mRNAs, the GO function and KEGG pathway annotation of differentially methylated genes were analyzed. GO analysis revealed that the hypermethylated genes were significantly enriched *in utero* embryonic development, protein stabilization, negative regulation of angiogenesis, and embryonic digit morphogenesis (**Figure 5A**). In contrast, the hypomethylated genes were significantly enriched in protein transport, cell cycle, cell division, and positive regulation of intracellular protein transport (**Figure 5B**). KEGG pathway analysis showed that the hypermethylated genes were significantly correlated with the MAPK signaling pathway, Hedgehog signaling pathway, and cell cycle (**Figure 5C**), whereas the hypomethylated genes were related to endocytosis, herpes simplex virus one infection, and thermogenesis (**Figure 5D**).

RNA-Seq and Combined Analysis of m6A MeRIP-Seq and RNA-Seq Results

On comparing the RNA-seq data of the two groups, 935 differentially expressed genes (fold change ≥1.5 and $p < 0.05$)

TABLE 3 | Sequencing reads and alignment statistics in placental of control and model rats.

Sample_ID	Raw reads	Valid reads	Valid%	Q20%	Q30%	GC%
Control-1 IP	55148418	54034460	90.55	97.71	93.45	51.70
Control-1 input	50843246	50127664	90.96	97.91	93.83	51.07
Control-2 IP	55352890	54347860	91.13	97.68	93.35	52.25
Control-2 input	55089734	54266690	91.18	97.82	93.63	52.17
Control-3 IP	55978166	54924124	90.84	97.67	93.34	51.83
Control-3 input	52129688	51397122	91.12	97.83	93.64	51.42
Model-1 IP	46581006	45716122	91.14	97.65	93.25	51.19
Model-1 input	54677212	53943366	91.32	97.76	93.49	50.24
Model-2 IP	52450058	51449630	91.01	97.80	93.65	51.34
Model-2 input	53611946	52843856	91.37	97.79	93.59	50.78
Model-3 IP	49856318	48983724	91.26	97.75	93.48	52.07
Model-3 input	54761116	54051736	91.81	97.84	93.66	51.20

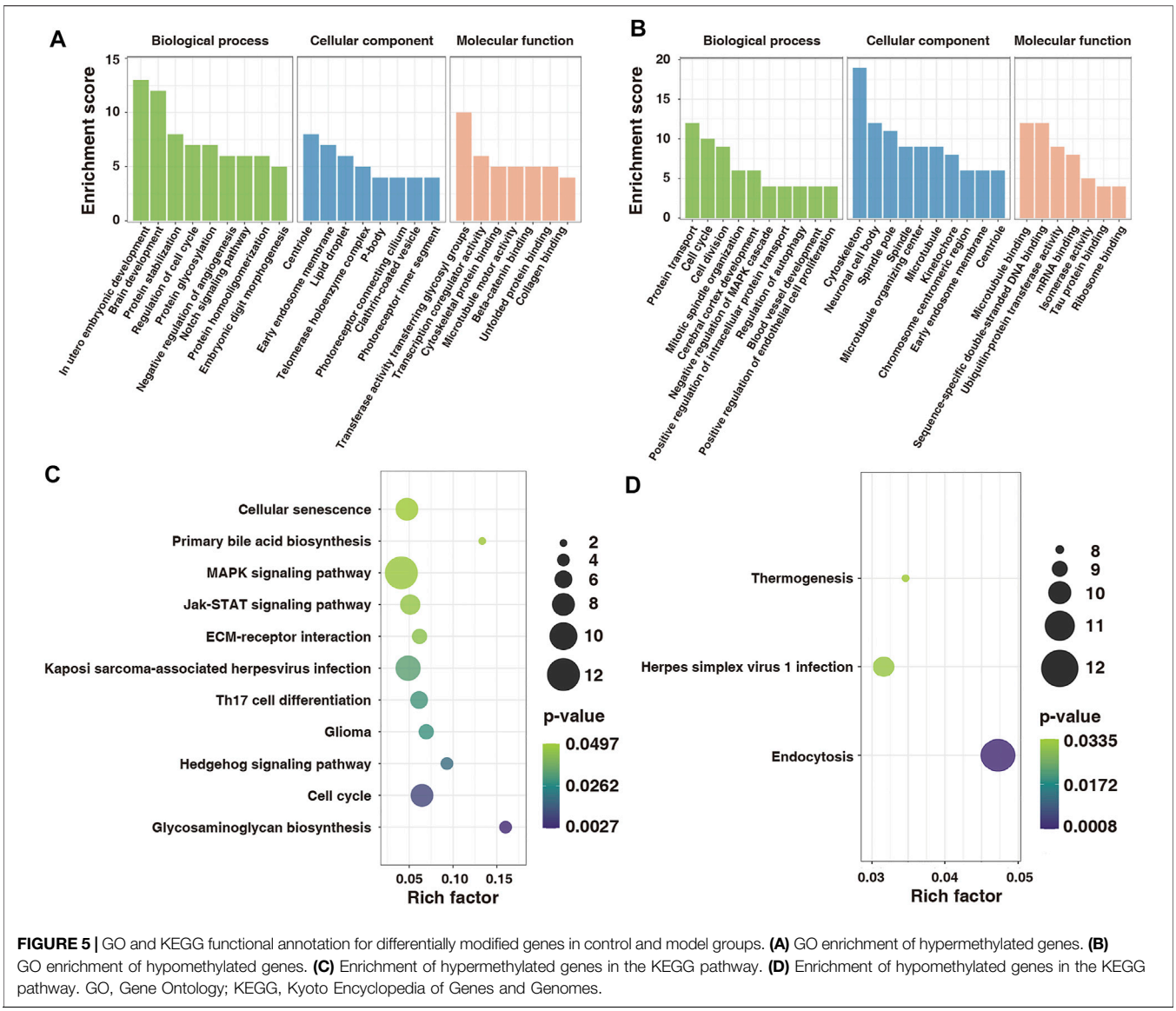

FIGURE 5 | GO and KEGG functional annotation for differentially modified genes in control and model groups. **(A)** GO enrichment of hypermethylated genes. **(B)** GO enrichment of hypomethylated genes. **(C)** Enrichment of hypermethylated genes in the KEGG pathway. **(D)** Enrichment of hypomethylated genes in the KEGG pathway. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

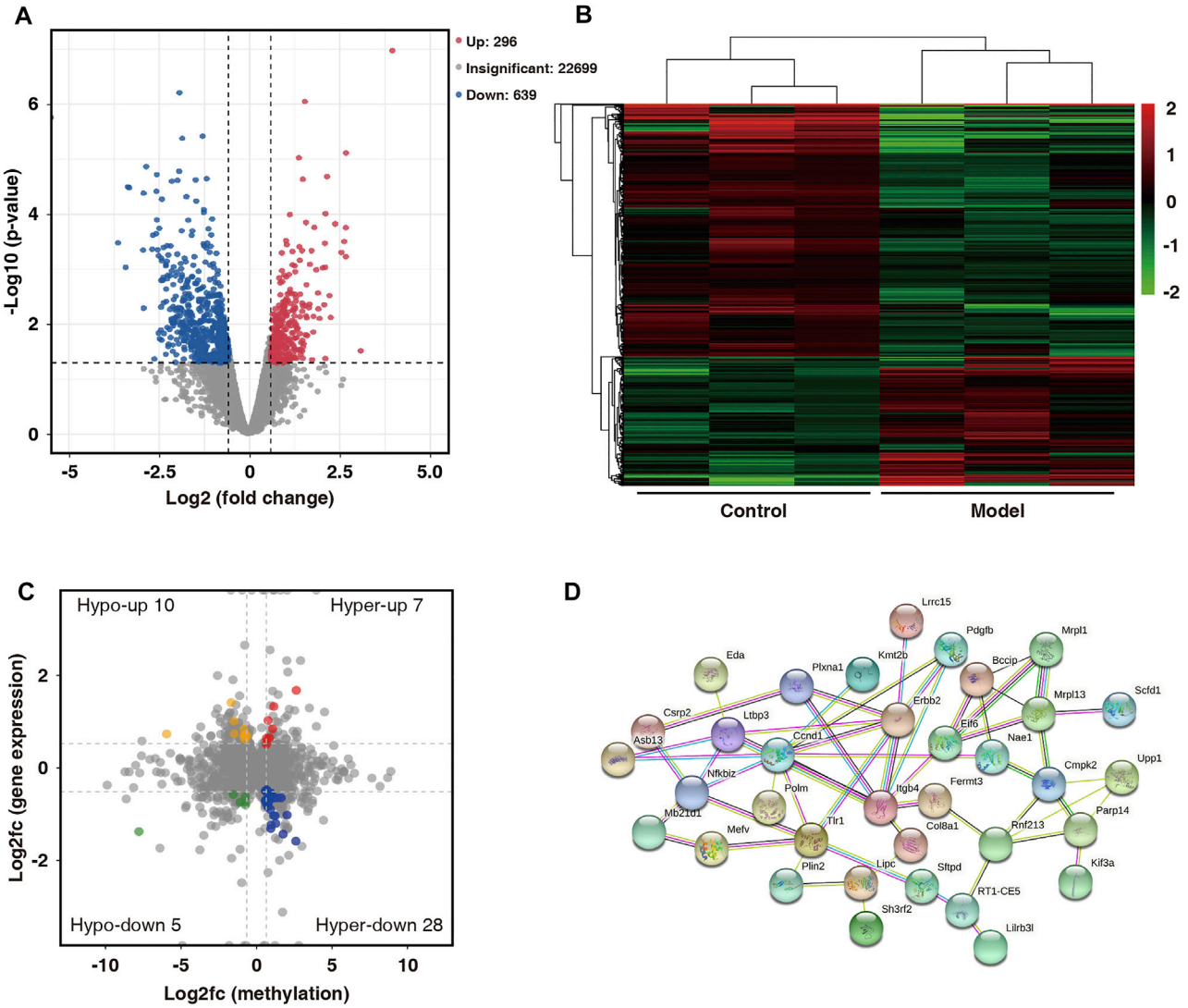

FIGURE 6 | Combined analysis of MeRIP-seq and RNA-seq data for control and model group. **(A)** Distribution of differentially expressed mRNAs in control and model groups (fold change ≥1.5 and $p < 0.05$). **(B)** Hierarchical clustering analysis of differentially expressed mRNAs. **(C)** Differentially modified and differentially expressed genes ("double aberrant genes") in control and model groups (fold change ≥1.5 and $p < 0.05$). **(D)** PPI network analysis of 50 double aberrant genes.

were found. Among them, 296 were upregulated and 639 were downregulated. The volcano map shows significantly different gene expressions between the two groups (**Figure 6A**). Subsequently, the 935 differentially expressed genes were used in hierarchical cluster analysis to identify differentially expressed genes in the two groups of samples (**Figure 6B**). By cross-analysis of the MeRIP-seq and RNA-seq data, 50 double aberrant genes were identified and divided into four groups: 7 "hyper-up" genes (highly methylated and overexpressed), 28 "hyper-down" genes (highly methylated and underexpressed), 10 "hypo-up" genes (hypomethylated and overexpressed), and 5 "hypo-down" genes (hypomethylated and underexpressed) (**Figure 6C**).

Verification of m6A Methylation Status and Gene Expression of Specific Genes

Protein–protein interaction (PPI) network analysis was performed on 50 double aberrant genes. A total of 27 double aberrant genes were found to have two or more nodes (**Figure 6D**; **Table 4**). *Mefv* and *Erbb2*, the highest and lowest node genes, were selected according to fold change. In addition, to confirm the accuracy of the sequencing results, we selected the non-node gene *Cgas* for verification experiments. MeRIP-qPCR and RT-qPCR were used to verify the m6A modification and mRNA expression of the three genes. *Mefv* showed hypermethylation and downregulated mRNA expression (**Figures 7A–C**), *Erbb2* showed hypomethylation and downregulated mRNA expression (**Figures 7D–F**), and *Cgas*

TABLE 4 | Double aberrant genes in PPI network.

Chromosome	Gene name	Peak start	Peak end	log2 (fold change)	*p*	m6A modification level
chr10	*Mefv*	12048873	12049321	2.6	0.01	up
chr11	*Col8a1*	45004814	45005172	2.02	0.02	up
chr14	*Tlr1*	45064237	45064564	1.76	0.01	up
chr11	*Parp14*	68135559	68136457	1.62	0.00	up
chr8	*Lipc*	77272600	77276649	1.14	0.03	up
chr1	*Fermt3*	222271705	222272285	1.13	0.01	up
chr4	*Plxna1*	121217659	121217809	1.13	0.00	up
chr20	*RT1-CE5*	4896430	4896991	1.05	0.01	up
chr7	*Pdgfb*	121214957	121215137	0.997	0.00	up
chr6	*Cmpk2*	45694642	45694822	0.94	0.00	up
chr5	*Plin2*	104984414	104984594	0.924	0.00	up
chr10	*Itgb4*	104560303	104560362	0.74	0.03	up
chr1	*Kmt2b*	89037521	89038195	0.673	0.00	up
chr1	*Ccnd1*	218090750	218091018	0.661	0.00	up
chr16	*Sftpd*	18753673	18753823	0.643	0.00	up
chr1	*Ltbp3*	221115887	221116096	0.59	0.00	up
chr3	*Eif6*	151356868	151356985	0.586	0.00	up
chr7	*Mrpl13*	95288406	95293667	–0.611	0.00	down
chr11	*Nfkbiz*	47270325	47270564	–0.741	0.00	down
chr1	*Bccip*	205777455	205783608	–0.742	0.00	down
chr7	*Csrp2*	53630621	53630680	–0.773	0.01	down
chr17	*Asb13*	70216354	70217243	–0.859	0.01	down
chr10	*Rnf213*	108579105	108579315	–1.1	0.03	down
chr19	*Nae1*	653510	659518	–1.47	0.00	down
chr14	*Mrpl1*	14982671	15008090	–1.49	0.00	down
chr14	*Upp1*	89314266	89314446	–1.54	0.02	down
chr10	*Erbb2*	86390165	86390803	–7.78	0.00	down

showed hypermethylation and upregulated mRNA expression (**Figures 7G–I**). These results were consistent with the sequencing data.

DISCUSSION

In this study, we performed the high-throughput sequencing of the placenta obtained from rat models of fear stress and found that fear stress during pregnancy can alter the levels of four m6A enzymes (METTL3, METTL14, WTAP, and FTO) as well as the levels of methylation modifications in the placenta, thereby revealing a specific m6A methylation profile for placenta with injury induced by fear stress during pregnancy. Some double aberrant genes were also found, which may be related to the regulation of placental function. Therefore, we infer that m6A modifications may regulate the expression levels of these genes and may play an essential role in the way fear stress response affects placental development.

Fear, a common psychological stress, is unlikely to cause disease in a short time or at low intensity. Nevertheless, long-term fear stimulation beyond physical adaptability and tolerance will lead to stress injury. Our group's previous research found that fear stress can lead to placental dysfunction and adverse fetal outcomes. The placenta has always been regarded as a critical link between the mother and fetus. It is well known that placental damage can lead to adverse fetal outcomes (Sun et al., 2020). In this study, we found that fear stress can reduce the number of live births, the total weight of the placenta, and the weight of the individual placenta.

M6A modification is a dynamic and reversible process that is involved in a variety of biological processes. Not only can affect mRNAs encoding histone modifiers and transcription factors but also chromatin-associated regulatory RNAs (Wei and He, 2021). Many studies related to m6A have been conducted in mammals, plants, and yeast. Previous studies in animals have shown that fear stress can modulate the expression of methylase in different regions of the brain, and can thereby affect m6A modification levels. Exposure to specific stressors and glucocorticoid levels can alter METTL3 and FTO expression in adult neurons, thereby affecting m6A modification levels and ultimately causing synaptic plasticity and increasing fear memory. Fear stress also decreased FTO levels and increased m6A modification levels in the neurons of the dorsal CA1 region of the hippocampus (Walters et al., 2017). Knocking down FTO in the medial prefrontal cortex of mice was found to increase m6A modification levels, which was associated with increased fear memory in mice (Widagdo et al., 2016).

However, there is a knowledge gap in regards to whether fear stress can alter methylation levels and methylesterase expression in the placenta. This study showed that fear stress during pregnancy can increase the overall levels of m6A modification in the placenta and alter the expression levels of METTL3, METTL14, WTAP, and FTO in the placenta. This critical finding provides an entry point for future studies on the

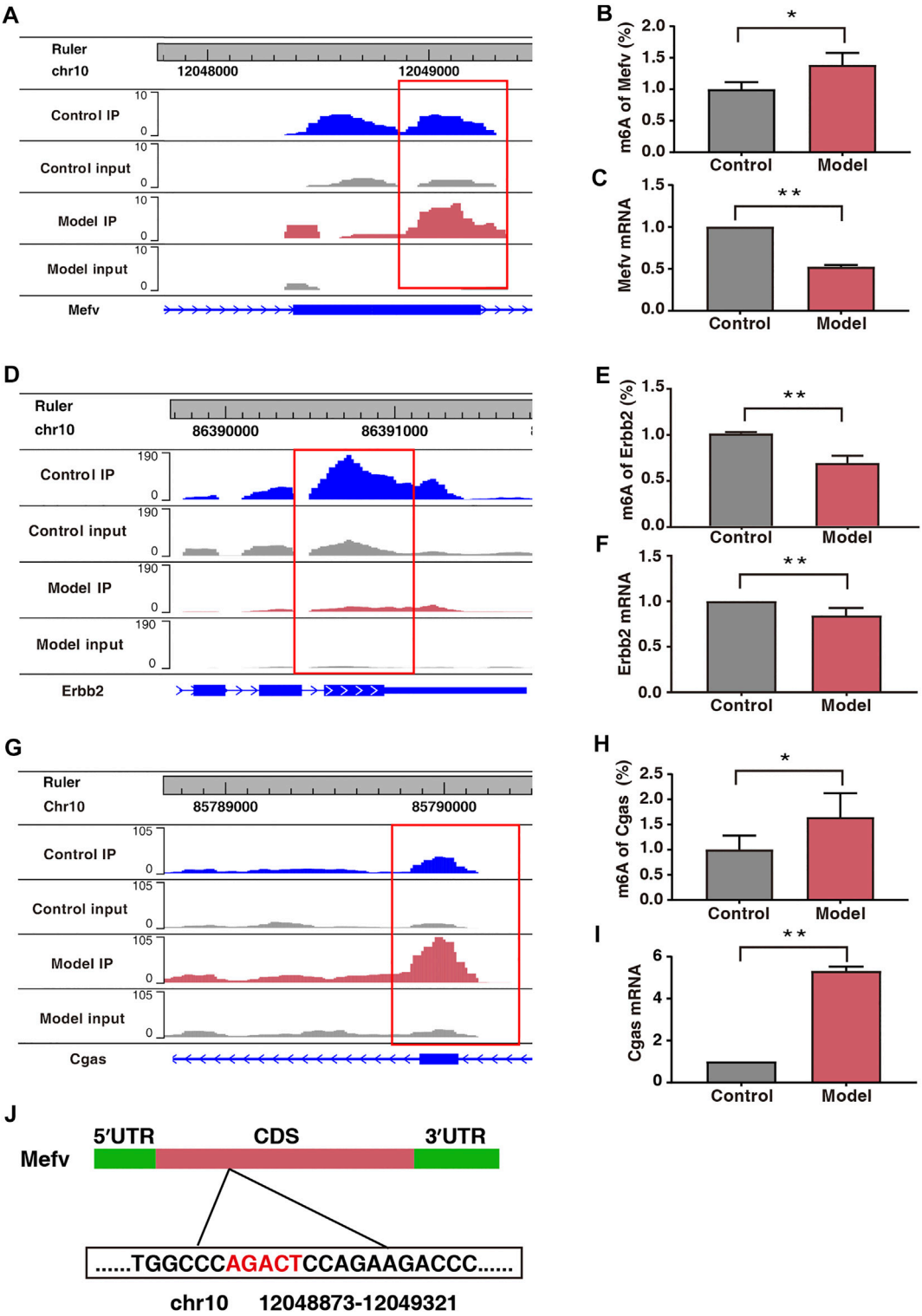

FIGURE 7 | Expression of *Mefv*, *Erbb2*, and *Cgas* was regulated by m6A modification. **(A)** Data visualization analysis of *Mefv* mRNA m6A modification in the two groups. **(B)** m6A modification levels of *Mefv* at a particular site ($n^* = 6$, $^{**}p < 0.05$). **(C)** mRNA levels of *Mefv* in the two groups ($n^* = 6$, $^{**}p < 0.01$). **(D)** Data visualization analysis of *Erbb2* mRNA m6A modification in the two groups. **(E)** m6A modification levels of *Erbb2* at a particular site ($n^* = 6$, $^{**}p < 0.01$). **(F)** mRNA levels of *Erbb2* in the two groups ($n^* = 6$, $^{**}p < 0.01$). **(G)** Data visualization analysis of *Cgas* mRNA m6A modification in the two groups. **(H)** m6A modification levels of *Cgas* at a particular site ($n^* = 6$, $^{**}p < 0.05$). **(I)** mRNA levels of *Cgas* in the two groups ($n^* = 6$, $^{**}p < 0.01$). **(J)** Schematic representation of the location of m6A modification site in *Mefv*.

relationship between epigenetic modifications and placental dysplasia induced by fear stress.

The specific increase in m6A modification levels in the placentas of rat models of fear stress may be associated with the altered expression of the methylation enzymes METTL3, METTL14, WTAP, and FTO. METTL3 is a core component of the catalytic subunit and is involved in cell development, cellular homeostasis, and cellular recoding (Liu et al., 2014). The primary role of METTL14 is to expand the range of RNA substrate recognition and enhance catalytic efficiency (Wang et al., 2016). METTL3 and METTL14 play essential roles in placental development and function. In the placentas of patients with eclampsia, both METTL3 and METTL14 are upregulated. METTL3 prevents trophoblast proliferation, migration, and invasion by promoting the recognition of pri-miR-497-5p/195-5p by DGCR8 (Wang J. et al., 2020). In our study, the expression levels of METTL3, METTL14, and WTAP in the placentas of rat models of fear stress were significantly increased, which corroborated the findings of the previously mentioned study. FTO was the first enzyme found to have an efficient oxidative demethylation activity (Jia et al., 2011), and its functional role in regulating placental and fetal development has received widespread attention. A study revealed that FTO expression was negatively correlated with placental quality in primiparous pregnancies and positively correlated with fetal weight and length (Bassols et al., 2010). Another study found that elevated FTO expression levels in the placenta were associated with an increased transport capacity of specific amino acid transport proteins, thereby ensuring efficient nutrient transport and promoting faster head size growth and increased fetal length in newborns (Barton et al., 2016). Notably, FTO expression was reduced and m6A modification was increased in the placental tissues of piglets with low birth weight; this was theorized to affect the expression of angiogenic and lipid metabolism genes in the placenta (Song et al., 2018). FTO knockout female mice exhibit significant ovarian defects and impaired fertility, which is associated with the involvement of m6A modification in regulating oocyte maturation and embryonic development. The absence of FTO caused increased m6A levels of LINE1 and decreased RNA levels of LINE1 in oocytes and embryos, while further indicating that the FTO-LINE1 RNA axis is functionally relevant in oocyte and embryo development in the mouse. This observation also indicates the prominent role of FTO-mediated m6A modifications in early embryonic development (Wei et al., 2022). In our study, the expression of placental FTO was significantly decreased due to fear stress. Correlation analysis showed that the expression of FTO was positively correlated with fear in pregnant rats and negatively correlated with the number of live births and the weight of the placenta, suggesting that FTO plays a major role as a core regulator of the imbalance in placental m6A modification caused by fear stress during pregnancy.

The RRACH motif is a classical sequence involved in m6A modification (Tao et al., 2017; Fitzsimmons and Batista, 2019), and from our experimental results, a common GGACU sequence was identified in both groups. This also supports the plausibility that m6A modifications occur in both groups. To investigate the potential functions of m6A-modified genes, GO and KEGG enrichment of differentially methylated peaks were performed. The results of GO enrichment revealed that the m6A-modified genes were mainly associated with angiogenesis, vascular endothelial cell development, protein transport, and cell cycle. The results of KEGG analysis revealed that the m6A-modified genes were mainly enriched in the MAPK signing pathway, Hedgehog signing pathway, and other signaling pathways. The MAPK signing pathway, a classical pathway, is involved in the regulation of embryonic development, placental development, vascular development, and other biological processes. In our study, the 12 aberrantly modified genes induced by fear stress were enriched in this pathway. It was previously reported that GPR4 can mediate oocyte maturation and regulate trophoblast infiltration and invasion via MAPK signaling (Qi et al., 2021). Moreover, PGF2α can reportedly promote angiogenesis in the porcine endometrium by activating the MAPK signaling pathway (Kaczynski et al., 2020). The overexpression of ERBB4 can also enhance vascular development via MAPK signaling (Liang et al., 2019). The Hedgehog signing pathway, which is involved in the induction of placental endoderm cell production, is closely associated with placental development and function and has also been prominently implicated in the etiology of PE (Jiang and Herman, 2006; Huang et al., 2021). The downregulation of the Hedgehog signaling pathway inhibits translation in the epithelial mesenchyme, attenuates trophoblast invasion and migration, and induces PE (Chen et al., 2019). In our study, genes with differential m6A modification were significantly enriched in these two pathways. Thus, m6A modification may regulate gene expression via the MAPK and Hedgehog signaling pathways. Further validation of these two pathways could help reveal new gene regulation mechanisms by which fear stress affects placental development at the mRNA level.

In our study, 50 double aberrant genes were identified by combining MeRIP-seq and RNA-seq data. These may be the target genes involved in placental m6A modification imbalance caused by fear stress. Three genes, *Mefv*, *Erbb2*, and *Cgas*, were selected for further analysis based on String results and in combination with low *p* and high FC values. We found elevated m6A modification levels and decreased mRNA expression levels of *Mefv* in the placental tissues of model rats. The decreased expression of *Mefv* can be attributed to the degradation function of m6A modification. Moreover, the reader proteins YTHDF1-3, FMR1, and HNRNPA2B1 were found in the core of mammalian stress granules (Jain et al., 2016). Recently, it has been found that the binding protein FMR1 can preferentially bind to mRNA containing m6A marker and "AGACU" motif and participate in the degradation of target mRNA by utilizing m6A modification (Zhang et al., 2022). In this study, RNA-seq analysis revealed that the binding protein FMR1 was significantly expressed in the placental tissues of model rats, and further sequence resolution of the m6A-modified region of *Mefv* revealed the sequence "AGACT (T = U)" (**Figure 7J**; **Table 5**). Therefore, we believe that FMR1 recognizes the upregulation of m6A modification induced by fear stress, thereby triggering degradation of its mRNA and ultimately leading to reduced expression of *Mefv*. Abnormal changes in

TABLE 5 | Expression of m6A methylation regulator.

Gene name	Chromosome	log2 (fold change)	*p*	Expression level
Ythdf3	chr2	0.25	0.39	up
Ythdf1	chr3	−0.24	0.43	down
Ythdc2	chr1	0.17	0.73	up
Ythdf2	chr5	0.05	0.77	up
Ythdc1	chr14	−0.06	0.98	down
Fmr1	X	0.95	0.00	up
Lrpprc	chr6	−0.25	0.42	down
Igf2bp1	chr10	−0.28	0.50	down
Alyref	chr10	−0.21	0.55	down
Elavl1	chr12	0.10	0.61	up

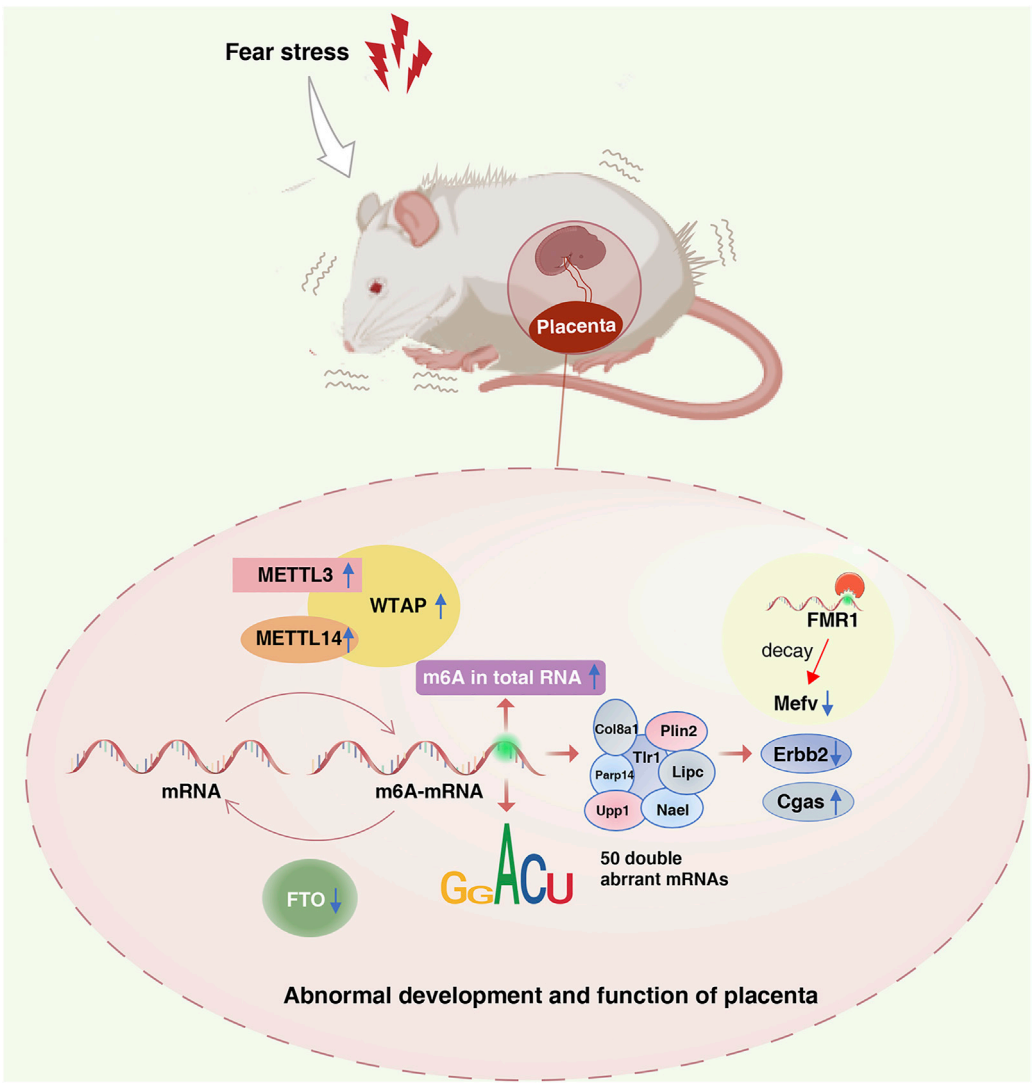

FIGURE 8 | Role of the m6A modification in placental dysfunction induced by fear stress during pregnancy. Fear stress during pregnancy increases the levels of methylated enzymes (METTL3, METTL14, and WTAP), decreases the levels of demethylase FTO, and increases the overall methylation levels in the placenta of pregnant rats. Fear stress during pregnancy can lead to abnormal modification and differential expression of 50 genes. Decreased expression of Mefv may rely on the decay function of m6A modific.

Mefv can cause familial Mediterranean fever (FMF) (Grandemange et al., 2011; Kirectepe et al., 2011), and there has been extensive research on *Mefv* in FMF. However, there remains a gap in research on stress-induced placental injury. This study reported that the function of *Mefv* may not be limited to FMF. *Erbb2* was another node gene whose m6A modification and mRNA expression levels decreased significantly. The target binding protein of Erbb2 was not determined in this study. Erbb2 is an epidermal growth factor receptor family member (Niu and Carter, 2007; Sharma et al., 2021), and its upregulation can increase angiogenesis. Erbb2 is also closely related to many genes related to vascular development in the genome. The overexpression of Erbb2 inhibits the transcription of antiangiogenic factors (Sparc, Timp3, and Serpinf1) but induces the expression of angiogenic factors (Klf5, Tnfaip2, and Sema3c) (Beckers et al., 2005). However, Erbb2 has not been reported to be associated with placental injury induced by fear stress during pregnancy.

In this study, *Mefv* and *Erbb2* were abnormally methylated and differentially expressed in the placentas of rat models of fear stress. PPI network analysis also showed that *Mefv* and *Erbb2* were involved in regulating several gene clusters. Therefore, we speculated that these two genes have excellent research potential as the target genes of fear stress that alter m6A modification in the placenta and lead to placental injury.

In summary, this study analyzed the effects of fear stress on enzyme methylation and methylation modification levels in the placenta, suggesting that m6A modification plays a role in placental dysfunction induced by fear stress during pregnancy (**Figure 8**). MeRIP-seq was used to sequence the placental tissues, providing a basis for revealing the functional mechanism of placental injury caused by fear stress during pregnancy based on m6A modification levels. Nevertheless, there are some limitations of our study. We determined that fear stress during pregnancy affects the expression of methylase in the placenta and preliminarily identified some differentially expressed genes. However, as we did not perform targeted inhibition or activation of specific methylases, we cannot determine the specific function of each enzyme. In the future, we plan on exploring this aspect more and investigating this topic in greater depth.

CONCLUSION

This was the first study to investigate changes in placental m6A-modifying enzymes and modification levels causing adverse fetal outcomes due to fear stress during pregnancy. Fifty double aberrant genes were identified by comprehensive MeRIP-seq and RNA-seq data analyses. *Mefv*, *Erbb2*, and *Cgas* were selected for validation, and the validation results were consistent with the sequencing data. We speculate that an imbalance in placental m6A modification is one of the potential pathogenic mechanisms by which fear stress during pregnancy causes placental damage. These double aberrant genes may be the target genes of placental dysfunction caused by fear stress during pregnancy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA837736.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Henan University of Chinese Medicine (permit number: DWLL2018030017).

AUTHOR CONTRIBUTIONS

QW conceived and designed the analysis and wrote the manuscript; MP collected the data and wrote the manuscript; LY and JH provided financial support, guided experimental design, and revised the manuscript; XL and PZ contributed analysis tools; AG helped in creating figures in the manuscript; and TZ and YJ conducted the experiments. All authors read and approved the final manuscript.

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Parental High-Fat High-Sugar Diet Intake Programming Inflammatory and Oxidative Parameters of Reproductive Health in Male Offspring

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Parental nutrition can impact the health of future generations, programming the offspring for the development of diseases. The developing germ cells of the offspring could be damaged by the maternal or the paternal environment. The germ cells in development and their function could be affected by nutritional adversity and therefore, harm the health of subsequent generations. The paternal or maternal intake of high-fat diets has been shown to affect the reproductive health of male offspring, leading to imbalance in hypothalamic-pituitary-gonadal axis, testicular oxidative stress, low testosterone production, and changes in sperm count, viability, motility, and morphology. There is a need for studies that address the combined effects of diets with a high-fat and high-sugar (H) content by both progenitors on male reproduction. In this context, our study evaluated epigenetic parameters and the inflammatory response that could be associated to oxidative stress in testis and epididymis of adult offspring. 90 days-old male rats were divided according to the combination of the parental diet: CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet).We evaluated serum levels of testosterone and FSH; testicular gene expression of steroidogenic enzymes *Star* and *Hsd17b3* and epigenetic markers *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Mecp2*; testicular and epididymal levels of TNF-α, IL-6, IL-10, and IL-1β; testicular and epididymal activity of SOD, CAT, and GST; the oxidative markers MDA and CP; the daily sperm production, sperm transit time, and sperm morphology. Testicular epigenetic parameter, inflammatory response, oxidative balance, and daily sperm production of the offspring were affected by the maternal diet; paternal diet influenced serum testosterone levels, and lower daily sperm production was exacerbated by the interaction effect of both parental intake of high-fat high-sugar diet in the testis. There was isolated maternal and paternal effect in the antioxidant enzyme activity in the cauda epididymis, and an interaction effect of both parents in protein oxidative marker. Maternal effect could also be observed in cytokine production of cauda epididymis, and no morphological effects were observed in the sperm. The potential programming effects of isolated or combined intake of a high-fat

high-sugar diet by the progenitors could be observed at a molecular level in the reproductive health of male offspring in early adulthood.

Keywords: testis, epididimys, epigenetics, high-fat diet, fetal programming, inflammation, oxidative stress, sperm production

1 INTRODUCTION

Inadequate nutrition through the intake of high-fat and high-sugar diets can set adverse issues for the health as an individual, and also for the health of next generations. According to the Developmental Origins of Health and Disease (DOHaD), adverse events in early stages of embryonic development could program the pattern of health and disease during the life of the offspring, a phenomenon known as fetal programming (Barker, 1998; Jazwiec and Sloboda, 2019). Parental inadequate nutrition has been shown to program the offspring for the development of diseases, given that the proper development and maturation of organs and systems in the early stages of life relies on healthy environments (Barker, 1998; Fleming et al., 2018; Jazwiec and Sloboda, 2019). Fetal programming can result from epigenetic modifications, that consist in heritable changes in gene expression with no alteration in DNA nucleotide sequence, such as covalent modifications in histone lysine residues, DNA methylation, and post-transcriptional changes (Reid et al., 2017; Safi-Stibler and Gabory, 2020). The parental intake of high-fat and high-sugar diets has been shown to influence the development of long-term chronic diseases such as cardiovascular disease, insulin resistance, hypertension and obesity in the offspring through epigenetics mechanisms (Fleming et al., 2018; Radford, 2018; Jazwiec and Sloboda, 2019).

The germ cells in development could also be affected by nutritional adversity, and experimental data reveals that parental intake of high-fat diets could harm the quality of reproductive parameters in both female and male offspring (Crean and Senior, 2019; Jazwiec and Sloboda, 2019; Sertorio et al., 2021). This issue becomes even more relevant due to the fact that offspring gametes of poor quality could still impair the metabolic health of subsequent generations (Radford, 2018). Parental high-fat diet intake prior to conception and/or during gestation and lactation seem to influence the reproductive health of male offspring (Sertorio et al., 2021). Adipose tissue hypertrophy leads to the production of adipokines, unbalancing the hypothalamic-pituitary-gonadal axis and impairing the production of gonadotropins (Jacobs et al., 2014; Rodríguez-González et al., 2015; Sanchez-Garrido et al., 2018); this impairment could then drive changes in the gene expression of steroidogenic acute regulatory protein (StAR) and 17β-hydroxysteroid dehydrogenase enzyme (17β-HSD), key steroidogenic enzymes of the testosterone production cascade (Sanchez-Garrido et al., 2018). The parental intake of high-fat diets could also program testicular oxidative imbalance in the offspring, disrupting the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione, with the production of cell damage markers, such as malondialdehyde (MDA) (Rodríguez-González et al., 2015; Bautista et al., 2017), harming testosterone production and spermatogenesis (Christante et al., 2013; Reame et al., 2014). Consequently, the sperm count, viability and motility, besides sperm morphology in the epididymis could be compromised (Fullston et al., 2015; Rodríguez-González et al., 2015). Changes in molecular sperm parameters could predict lower sperm quality and therefore, poor development of a potential zygote, setting risk for more than one generation (Palmer et al., 2012; Fullston et al., 2013; Fleming et al., 2018).

Adipose tissue accumulation high-fat diet-induced can be related to altered DNA methylation profile in the germline of adult male rats, such as altered expression pattern of DNA methyltransferases (DNMTs) in the testis (Deshpande et al., 2020). It can also be related to the development of an inflammatory state in the male genital tract, with the production of the cytokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β), and interleukin 10 (IL-10) (Tremellen, 2016; Fan et al., 2018; Yi et al., 2020). The inflammatory response induced by adiposity could generate reactive oxygen species (ROS), overloading the antioxidant capacity in the testis, and reducing sperm quality (Yi et al., 2020). To our knowledge, this is the first programming study addressing epigenetic and inflammatory changes in the reproductive health of male offspring from progenitors fed on high-fat high-sugar diet, as well as the additive impact of high-fat diets intake by both progenitors on male reproduction. Our objective was to assess the parental high-fat high-sugar epigenetic programming on male reproductive health in testis and epididymis of adult rats, focusing on the link between the inflammatory response and oxidative stress over sperm production in the offspring.

2 MATERIALS AND METHODS

2.1 Animals and Diet

All animals were housed in polypropylene cages under controlled lighting (light/dark cycle) and temperature (22°C) with free access to its proper diet and drinking water. Male Wistar rats (8-weeks-old) were submitted to the intake of a control (n = 11) or a modified diet (n = 11), prior to conception during 10 weeks. Female Wistar rats (10-weeks-old) also received a control or modified diet during gestation and lactation. Males and females were randomly placed to receive control diet (CD) (15.5% fat; 50% carbohydrates, Nuvilab CR1, Quimtia®) or high-fat high-sugar diet (H) [32% fat; 50% carbohydrates (25% from sugar)]. Sweetened condensed milk was the main source of sugar and lard the main source of fat in H diet. Vitamin and mineral mix were added to avoid micronutrient deficiencies, as well as choline

bitartrate and L-cysteine in order to provide the quantity of essential nutrients of CD diet (César et al., 2021).

After 10 weeks of diet intake, males were mated to females. When copulation was confirmed, females were fed CD or H diet during gestation and lactation. The period of 10 weeks comprises a whole cycle of spermatogenesis for male rats, assuring this way, that all germ cells were exposed to the treatment. This period is also considered a chronic treatment for inducing increased adiposity in rats. Gestation is the most important period of growth and development, and maternal modified diet during this period has been shown to program the development of offspring. The following groups were formed: CD (control paternal and maternal diet, n = 6), HP (H paternal diet and control maternal diet, n = 5), HM (H maternal diet and control paternal diet, n = 5) and HPM (H paternal and maternal diet, n = 6). Eight male pups were used for each litter per dam, and after weaning, the male pups were fed a control diet during 90 days. 1–2 pups per litter were used in the experimental analyses. Each individual male offspring was treated as a biological replicate for statistics.

2.2 Euthanasia and Material Collection

Male progenitors were euthanized after mating, female progenitors after 21 days of weaning and male offspring at 90 days old. The animals were fasted for 10–12 h prior to euthanasia, and placed in anesthesia jar containing cotton soaked in isoflurane. The cotton pad was separated through a physical barrier to avoid direct contact between animal and the anesthetic. After confirmation of lack of reflex and reduced respiratory rate, animals were euthanized by decapitation under deep anesthesia. All the procedures were performed following the rules issued by the National Council for Control of Animal Experimentation (CONCEA). The use of animals for this study was approved by the Ethic Committee on Animal Use of the Federal University of São Paulo (CEUA number 9856031018/UNIFESP).

Trunk blood was collected and centrifuged at 700 x g in 4°C for 15 min. Serum was collected, frozen and stored (−80°C). Retroperitoneal (RET), mesenteric (MES), and epididymal (EPI) adipose tissue, testis, and epididymis were weighted, frozen and stored (−80°C). Sperm from the vas deferens was collected and fixed in 4% buffered formaldehyde for morphologic analysis.

2.3 Quantification of Serum Testosterone and FSH Levels

The quantification of serum levels of testosterone and FSH was performed through chemiluminescence assay by the clinical analysis laboratory Hermes Pardini, SP, Brazil.

2.4 Gene Expression of Steroidogenic Enzymes and Epigenetic Markers

Extraction of total RNA from testis samples was performed using trizol reagent (TRI-reagent, Sigma, St. Louis, MO, United States), according to the manufacturer's instructions. To obtain the concentration of RNA/μl, the reading was performed at wavelengths of 260, 280 and 230 nm. The degree of purity was estimated by the 260/280 nm ratio which must vary between 1.8 and 2.0 for nucleic acids. Total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, United States). For cDNA generation, the total RNA of each sample was subjected to reverse transcription, using the M- MLV Reverse Transcriptase Kit (PROMEGA, Madison, WI, United States). Relative levels of mRNA for *Star* and *Hsd17b3* in the testis were quantified using the PCR-RT technique using the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, United States). Primers sequences of Beta-actin, StAR and 17β-HSD were obtained from the U.S National Library of Medicine Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). To assess stability of the primers in the samples, amplification efficiency curves were determined. Gene sequences of DNA methyltransferase 1 (*Dnmt1*), DNA methyltransferase 3a (*Dnmt3a*), DNA methyltransferase 3b (*Dnmt3b*) and Methyl CpG binding protein 2 (*Mecp2*) were used according to previous study from our research group (Santamarina et al., 2018). Primer sequences used in the study are displayed in **Table 1**. The detection method used was the SyberGreen fluorophore (Applied Biosystems, Foster City, CA, United States). Relative mRNA levels for *Hprt* (hypoxanthine phosphoribosyltransferase-1) and Beta-actin were determined as reference genes. The information was recorded using the Sequence Detector software (Applied Biosystems, Foster City, CA, United States).

2.5 Extraction of Proteins in the Testis and Epididymis

Testis and epididymis samples (200 mg) were homogenized in 0.6 ml of extraction buffer (100 mM EDTA, 100 mM Tris, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 2 mM phenylmethylsulphonyl fluoride, and 0.1 mg/ml aprotinin. To the homogenized samples Triton X-100 10% was added and then centrifuged after 30 min at 22.600 × g for 40 min at 4°C in order to collect the supernatant. (César et al., 2021). The supernatant was used to quantify the cytokine levels and total protein content, according to the Bradford method (Bradford, 1976). Samples were kept at −80°C.

2.6 Levels of TNF-α, IL-6, IL-1β, and IL-10 in the Testis and Epididymis

The levels of the cytokines TNF-α, IL-6, IL-1β, and IL-10 in the testis and epididymis were determined using the ELISA method using R&D Systems kits, following the manufacturer's instructions. For detection limit, standard curves were calculated for each cytokine. The standard curve for TNF-α (DY510), IL-10 (DY522), and IL-1β (DY501) ranged from 62.5 to 4.000 pg/ml, and IL-6 (DY506) ranged from 125 to 8.000 pg/ml.

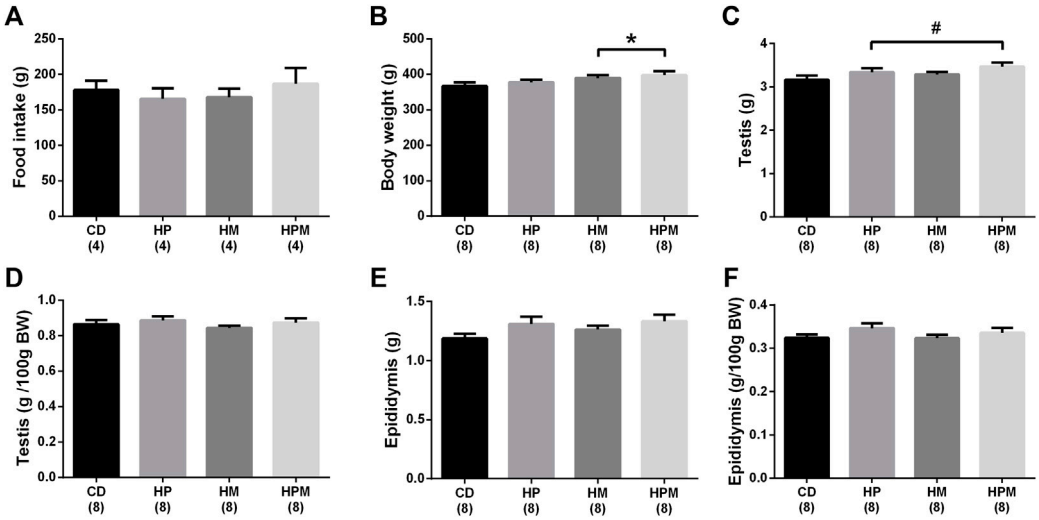

FIGURE 1 | Biometric data and food intake of male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): #statistically significant by the effect of paternal diet; *statistically significant by the effect of maternal diet. H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet); BW: body weight. **(A)**: food intake; **(B)**: body weight (g); **(C)**: testis (g); **(D)**: testis (g/100 g BW); **(E)**: epididymis (g); epididymis (g/100 g BW); ($n = 8$).

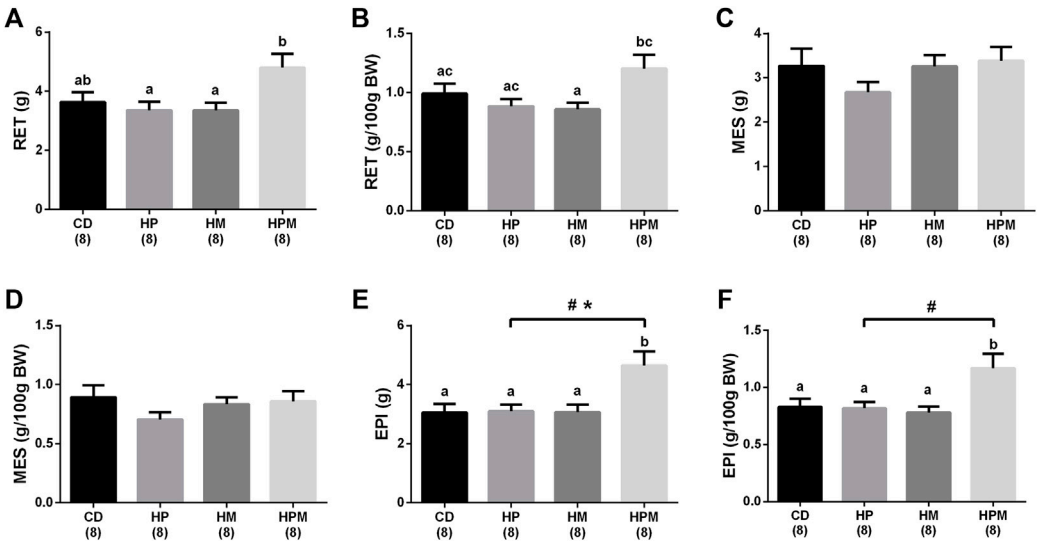

FIGURE 2 | Adipose tissue data of male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by the effect of parental diet ($p < 0.05$): #statistically significant by paternal diet; *statistically significant by the effect of maternal diet. a,b,c Different letters represent significant difference between groups ($p < 0.05$). H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet); BW: body weight; **(A)**: retroperitoneal adipose tissue (RET) (g); **(B)**: RET (g/100 g BW); **(C)**: mesenteric adipose tissue (MES) (g); **(D)**: MES (g/100 g BW); **(E)**: epididymal adipose tissue (EPI) (g); **(F)**: EPI (g/100 g BW); ($n = 8$).

2.7 Spermatid Number, Daily Sperm Production, Sperm Number and Transit Time

Spermatids resistant to homogenization in the testis and sperm resistant to homogenization in the caput/corpus and cauda epididymis were counted in Newbauer chambers (four fields per animal), after homogenization of the organ in a solution of 0.9% NaCl and 0.05% Triton X-100 (Robb et al., 1978).

To determine the daily sperm production, the number of spermatids per testis was divided by 6.1, which corresponds to the number of days in which mature spermatids are present in the seminiferous epithelium. For sperm transit time in the caput/corpus and cauda epididymis in days, the number of sperm in

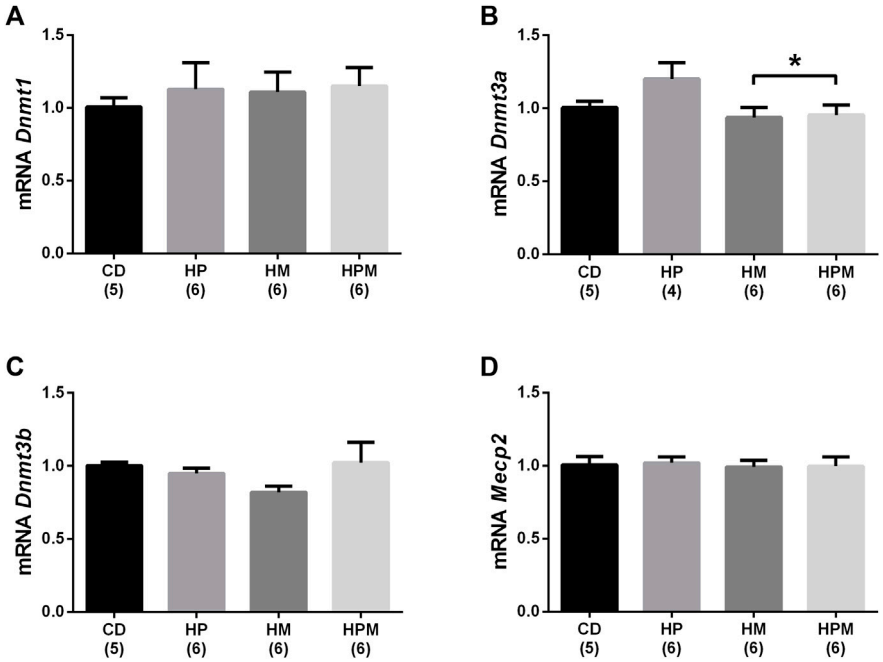

FIGURE 3 | Testicular gene expression of epigenetic markers in male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): *statistically significant by the effect of maternal diet. H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet). **(A)**: DNA methyltransferase 1 (*Dnmt1*) (n = 5-6); **(B)**: DNA methyltransferase 3a (*Dnmt3a*) (n = 4-6); **(C)**: DNA methyltransferase 3b (*Dnmt3b*) (n = 5-6); **(D)**: methyl CpG binding protein 2 (*Mecp2*) (n = 5-6).

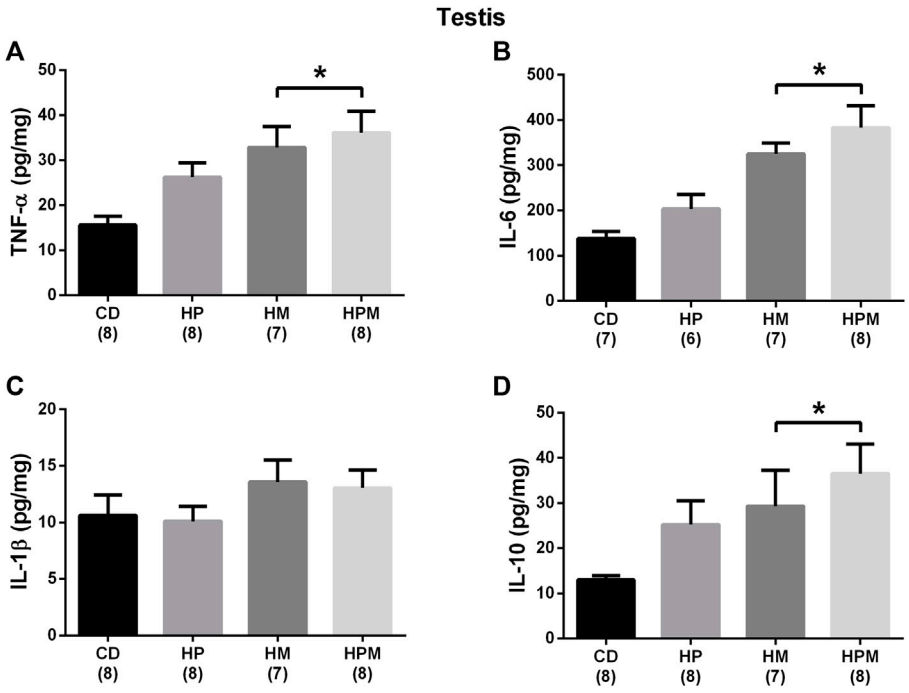

FIGURE 4 | Testicular cytokine levels in male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): *statistically significant by the effect of maternal diet. H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet). **(A)**: tumor necrosis factor α (TNF-α) (pg/mg) (n = 7-8); **(B)**: interleukin 6 (IL-6) (pg/mg) (n = 6-8); **(C)**: interleukin 1β (IL-1β) (pg/mg) (n = 7-8); **(D)**: interleukin 10 (IL-10) (pg/mg) (n = 7-8).

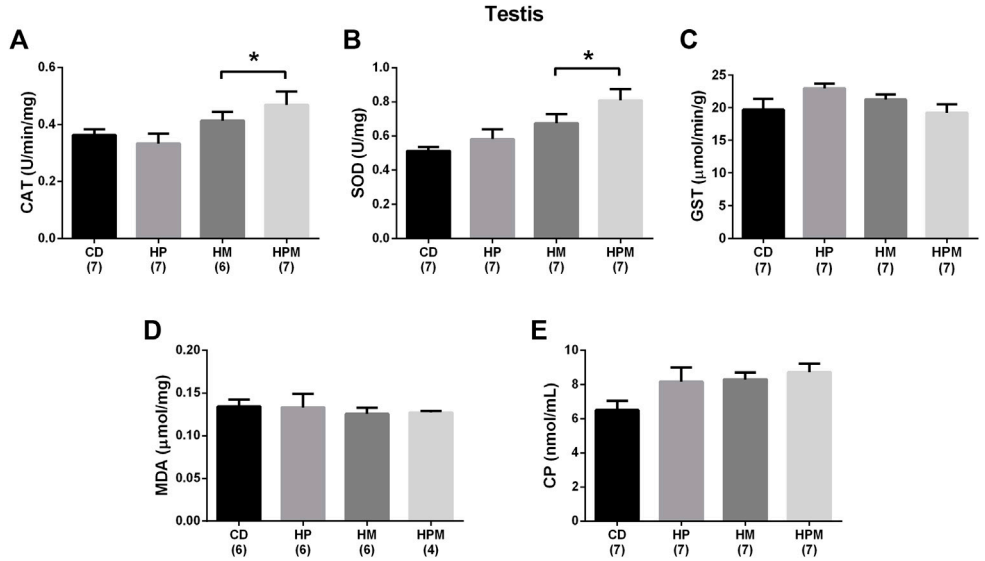

FIGURE 5 | Testicular antioxidant enzyme activity and oxidative stress markers in male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): *statistically significant by the effect of maternal diet. H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet). **(A)** catalase (CAT) (U/min/mg) ($n = 6$-7); **(B)**: superoxide dismutase (SOD) (U/mg) ($n = 7$); **(C)**: glutathione S-transferase (GST) (µmol/min/g) ($n = 7$); **(D)**: malondialdehyde (MDA) (µmol/mg) ($n = 4$-6); **(E)**: CP (carbonyl protein) (nmol/mL) ($n = 7$).

TABLE 1 | Primer sequences used in the study.

Genes	Sequences
Beta-actin F	CTAAGGCCAACCGTGAAAAGA
Beta-actin R	CCAGAGGCATACAGGGACAAC
Hprt F	CTCATGGACTGATTATGGACAGGAC
Hprt R	GACGGTCAGCAAGAACTTATAGCC
Star F	AAGGCTGGAAGAAGGAAAGC
Star R	CACCTGGCACCACCTTACTT
Hsd17b3 F	TGACCAAGACCGCCGATGAGTT
Hsd17b3 R	TGGGTGGTGCTGCTGTAGAAGAT
Dnmt1 F	TCCTACGCCATGCCCAGTTTG
Dnmt1 R	GAAGATGGGCGTCTCATCATCG
Dnmt 3a F	GCCCATTCGATCTGGTGATTG
Dnmt 3a R	TCGTAAAGTCCCTTGCGGGC
Dnmt 3b F	TGTGCAGAGTCCATTGCTGTAGGA
Dnmt 3b R	GCTTCCGCCAATCACCAAGTCAAA
Mecp2 F	CAGCTCCAACAGGATTCCATGGT
Mecp2 R	TGATGTCTCTGCTTTGCCTGCCT

F, forward; R, reverse.

each portion was divided by the daily sperm production (Robb et al., 1978).

2.8 Sperm Morphology

Sperm morphology was performed using sperm from spermatic fluid of the vas deferens and fixed in 4% buffered formaldehyde. The solution was applied to histological slides for analysis of two hundred sperm per sample under a Nikon Eclipse E100 microscope (Tokyo, Japan) in a 40x magnification lens. Sperm were classified as normal or with head or tail defects (Filler, 1993).

2.9 Antioxidant Enzyme Activity and Oxidative Stress Markers

Testis and epididymis samples (150 mg) were homogenized in phosphate buffer 0.2 M pH 7.4 (1 ml) and centrifuged at 10.000 × g at 4°C for 10 min. The supernatant was used for the analysis of CAT, SOD and glutathione S-transferase (GST) activities, MDA oxidative marker and total protein content. For the carbonyl protein (CP) oxidative marker, the pellet was used. CAT activity was estimated by measuring the decomposition of hydrogen peroxide (Góth, 1991). SOD activity was evaluated by the pyrogallol method, based on the ability of this enzyme to catalyze the reaction of superoxide and hydrogen peroxide. GST activity was estimated by the conjugation of gluthatione tiol groups to 1-chloro-2,4-dinitrobenzene (Habig et al., 1974) MDA was assessed by the formation of thiobarbituric acid reactive substances (Buege and Aust, 2007) and CP was determined by the derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine, leading to the formation of dinitrophenyl hydrazones (Levine et al., 1994). Total protein content was assessed according to the Bradford method (Bradford, 1976).

2.10 Statistical Analysis

The normality of the data was evaluated by Shapiro–Wilk test and logarithmic transformation was used for normalization of data.

TABLE 2 | Sperm parameters in testis and epididymis in male rat offspring from progenitors fed on high-fat and high-sugar diet.

Sperm parameters	CD (n = 6)	HP (n = 6)	HM (n = 6)	HPM (n = 6)
Spermatid number (x 10^6/testis)	202.4 ± 10.75^{a}	156.8 ± 7.13^{b}	137.6 ± 5.39^{b*}	158.1 ± 6.04^{b*}
Spermatid number (x 10^6/g testis)	142.2 ± 7.70^{a}	104.5 ± 6.19^{ab}	90.44 ± 4.40^{ab*}	101.9 ± 3.90^{b*}
Daily sperm production (x 10^6/testis/day)	33.18 ± 1.76^{a}	25.70 ± 1.17^{b}	22.56 ± 0.88^{b*}	25.91 ± 0.99^{b*}
Caput/corpus epididymis sperm number (x 10^6/organ)	106.9 ± 8.61	114.1 ± 11.38	95.21 ± 6.90*	85.11 ± 4.4*
Caput/corpus epididymis sperm number (x 10^6/g organ)	339.7 ± 23.64	345.8 ± 38.01	288.7 ± 17.56*	265.1 ± 15.01*
Sperm transit time in the caput/corpus epididymis (days)	3.237 ± 0.24	4.471 ± 0.47	4.215 ± 0.22	3.354 ± 0.30
Cauda epididymis sperm number (x 10^6/organ)	134.9 ± 6.00	139.9 ± 14.52	133.9 ± 12.03	138.5 ± 8.99
Cauda epididymis sperm number (x 10^6/g organ)	641.3 ± 30.39	635.4 ± 40.33	618.0 ± 55.06	653.8 ± 34.21
Sperm transit time in the cauda epididymis (days)	4.132 ± 0.30	5.449 ± 0.53	6.040 ± 0.73	5.431 ± 0.46
Normal shaped spermatozoa	178.0 ± 2.91	176.7 ± 2.75	180.8 ± 4.26	176.8 ± 3.73

^{a,b}Different letters represent significant difference between groups (p < 0.05).

*Mean ± SEM., Superscript symbols indicate significant difference caused by parental diet (p < 0.05): *statistically significant by the effect of maternal diet. H, high-fat and high-sugar diet; CD, (control paternal and maternal diet), HP, (H paternal diet and control maternal diet), HM, (H maternal diet and control paternal diet), and HPM, (H paternal and maternal diet).*

The identification of outliers was performed by the Grubbs method and data were assessed by t-test for parental data and two-way ANOVA, followed by Bonferroni post-hoc test for offspring data. Results were expressed as mean and the standard error of the mean (SEM). Statistical analysis was performed with the software JASP 0.12.1.0 with a significance level of $p < 0.05$. Results were described according to the effect of the factor causing the changes (paternal or maternal diet). Groups were compared individually by the post-hoc test when the interaction between the factors was detected.

3 RESULTS

3.1 Progenitors

3.1.1 Biometric Data

Significant changes caused by the high-fat high-sugar diet intake were observed in both male and female progenitors (**Supplementary Figure S1**). There was an increase in female progenitor body weight at the end of gestation ($n = 11$, $p < 0.05$, **Supplementary Figure S1A**), but not after the lactation period ($n = 11$, $p > 0.05$, **Supplementary Figure S1B**) when compared to the control group. There was also an increase in the relative weight of RET compared to CD ($n = 8$, $p < 0.05$, **Supplementary Figure S1C**). After 10 weeks of treatment, the body weight was increased in male progenitors in comparison to CD ($n = 11$, $p < 0.05$, **Supplementary Figure S1D**), as well as RET adiposity ($n = 8$, $p < 0.05$, **Supplementary Figure S1E**).

3.2 Male Offspring

3.2.1 Maternal Influence

Our results showed significant changes in biometric data, epigenetic pattern, inflammatory response, oxidative balance, and sperm count of male offspring from female progenitors fed on high-fat high-sugar diet. There was an isolated maternal effect of diet intake on the increase in offspring body weight ($n = 8$, $p < 0.05$, **Figure 1B**), which could be a reflex of the increased adiposity of EPI absolute weight ($n = 8$; $p > 0.05$, **Figure 2E**). Adiposity could be related to the decrease in *Dnmt3a* gene expression ($n = 4$–6; $p < 0.05$, **Figure 3B**) and to the increase of TNF-α, IL-6, and IL-10 levels in the testis ($n = 6$–8; $p < 0.05$; **Figures 4A,B,D**, respectively). Along with the cytokines, the antioxidant activity of CAT and SOD was increased ($n = 6$–7; $p < 0.05$, **Figures 5A,B**, respectively), showing the inflammatory response that could be associated to an oxidative imbalance. As a result, sperm count and daily sperm production in the testis and sperm count in caput/corpus in epididymis was decreased by the maternal effect (**Table 2**). Unlike the testis, there was a decrease in TNF-α levels in the epididymis ($n = 8$; $p < 0.05$, **Figure 6A**), with an increase in CAT levels ($n = 6$-7; $p < 0.05$, **Figure 7A**).

3.2.2 Paternal Influence

Paternal diet alone was able to influence the increase in absolute and relative weight of EPI ($n = 8$; $p > 0.05$, **Figures 2E,F**), along with the absolute testicular weight ($n = 8$, $p < 0.05$, **Figure 1C**). Increased adiposity is commonly associated to the decrease in serum testosterone levels ($n = 6$–8, $p < 0.05$, **Figure 8A**), as observed here in. The intake of a high-fat high sugar diet by the male progenitor decreased GST activity in the epididymis ($n = 7$; $p < 0.05$, **Figure 7C**), as well as the levels of the oxidative marker CP ($n = 5$–7; $p < 0.05$, **Figure 7E**).

3.2.3 Interaction Effect of Maternal and Paternal Diet Intake

There was an interaction effect of maternal and paternal diet intake on the increase in adiposity (**Figure 2**). The absolute weight of RET in the HPM group was increased by the interaction effect when compared to HP and HM ($n = 8$, $p < 0.05$, **Figure 2A**), and the relative weight was increased in HPM when compared to HM ($n = 8$, $p < 0.05$, **Figure 2B**). The interaction effect also increased the absolute and relative weight of EPI in HPM group when compared to all groups ($n = 8$; $p > 0.05$, **Figures 2E,F**, respectively), which points towards the synergistic effect resulted from both parental intake. The interaction effect of the progenitors decreased the number of spermatids in the testis in all groups when compared to control, the number of spermatids per g testis in HPM compared to control, and also decreased the daily sperm production in all groups when compared to control ($n = 5$–6; $p < 0.05$, **Table 2**). Ultimately, the interaction effect reduced CP levels in HPM group when compared to HM group ($n = 5$–7; $p < 0.05$, **Figure 7E**).

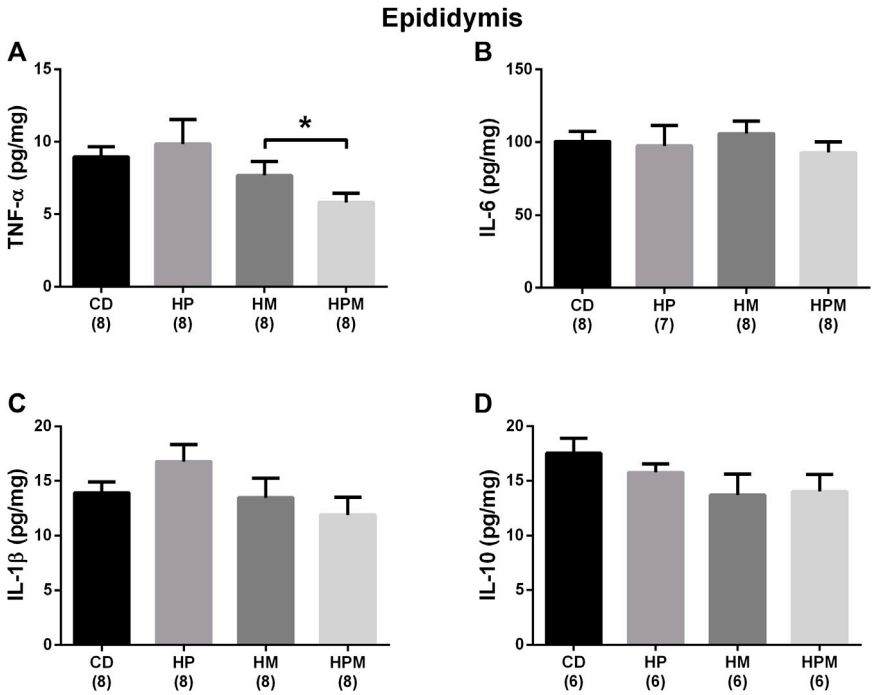

FIGURE 6 | Epididymal cytokine levels in male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): *statistically significant by the effect of maternal diet. H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet). A: tumor necrosis factor α (TNF-α) (pg/mg) ($n = 8$); B: interleukin 6 (IL-6) (pg/mg) ($n = 7$-8); C: interleukin 1β (IL-1β) (pg/mg) ($n = 8$); D: interleukin 10 (IL-10) (pg/mg) ($n = 6$).

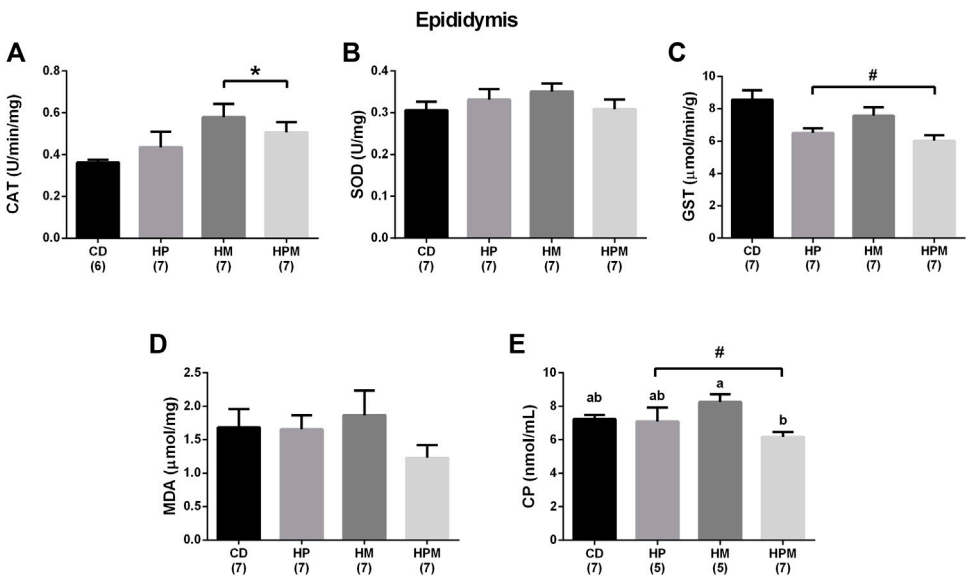

FIGURE 7 | Epididymal antioxidant enzyme activity and oxidative stress markers in male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): #statistically significant by the effect of paternal diet; *statistically significant by the effect of maternal diet. a,b Different letters represent significant difference between groups ($p < 0.05$). H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet). **(A)**: catalase (CAT) (U/min/mg) ($n = 6$-7); **(B)**: superoxide dismutase (SOD) (U/mg) ($n = 7$); **(C)**: glutathione S-transferase (GST) (μmol/min/g) ($n = 7$); **(D)**: malondialdehyde (MDA) (μmol/mg) ($n = 7$); **(E)**: carbonyl protein (CP) (nmol/mL) ($n = 5$-7).

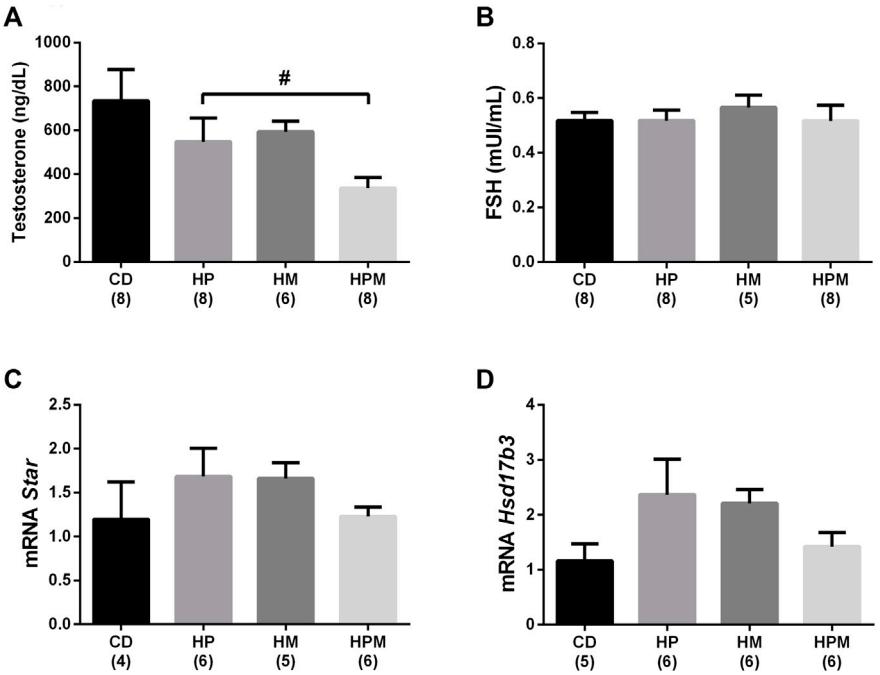

FIGURE 8 | Serum levels of sex hormones and gene expression of testicular steroidogenic enzymes in male rat offspring from progenitors fed on high-fat high-sugar diet. Mean ± SEM; symbols indicate significant difference caused by parental diet ($p < 0.05$): #statistically significant by the effect of paternal diet. H: high-fat and high-sugar diet; CD (control paternal and maternal diet), HP (H paternal diet and control maternal diet), HM (H maternal diet and control paternal diet) and HPM (H paternal and maternal diet). **(A)**: serum testosterone: $n = 6–8$; **(B)**: serum follicle stimulating hormone (FSH) ($n = 5–8$); **(C)**: steroidogenic acute regulatory protein (*Star*) ($n = 4–6$); **(D)**: 17β-hydroxysteroid dehydrogenase 3(*Hsd17b3*) ($n = 5–6$).

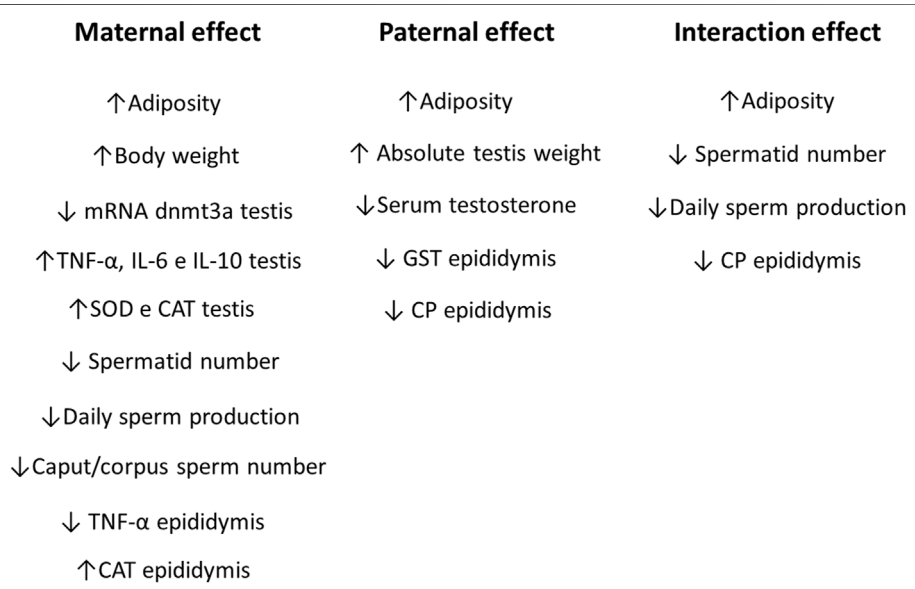

FIGURE 9 | Maternal, paternal, and interaction effects in male rat offspring from progenitors fed on high-fat and high-sugar diet.

Regarding the remaining results, there was no effect for food intake ($n = 4$), testicular relative weight, and absolute and relative epididymal weight ($n = 8$, $p > 0.05$, **Figures 1A,D–F**, respectively). No differences were observed for absolute and relative MES weight ($n = 8$; $p < 0.05$, **Figures 2C,D**) and no alterations were observed for the testicular gene expression of *Dnmt1*, *Dnmt3b*, and *Mecp2* ($n = 5–6$; $p < 0.05$, **Figures 3A,C,D**, respectively). In the testis, there were no changes for levels of IL-1β ($p > 0.05$, **Figure 4C**), GST ($n = 7$; $p > 0.05$,

Figure 5C) nor for the oxidative stress markers MDA and CP (n = 4–7; $p < 0.05$, **Figure 5D,E**, respectively).

In the epididymis, no changes were observed in the levels of IL-6, IL-1β, IL-10 (n = 6–8; $p > 0.05$, **Figures 6B–D**, respectively), SOD and MDA (n = 7; $p > 0.05$, **Figures 7B,D**, respectively). There were no changes in serum FSH (n = 5–8; $p > 0.05$; **Figure 8B**) nor testicular gene expression of star and hsd17b3 (n = 5–6; $p > 0.05$; **Figures 8C,D**, respectively). The results of male offspring are summarized in **Figure 9** according to the effect of each progenitor and their interaction.

4 DISCUSSION

Our study evaluated the effects of parental high-fat high-sugar diet intake on the reproductive health of adult male rats. The results showed that parental diet can program epigenetic parameters, adiposity, inflammatory response, and oxidative balance in the testis of adult rats, altering daily sperm production and serum testosterone levels. To our knowledge, this is the first study to evaluate epigenetic markers and cytokine production associated to oxidative stress and sperm parameters in the testis and epididymis of male adult offspring from both parents submitted to a high-fat high-sugar diet intake.

Epigenetics comprehends heritable changes in gene expression without changes in DNA nucleotide sequence. DNA methylation is an epigenetic marker that regulates gene expression through gene silencing, a process catalyzed by DNA methyltransferases (DNMTs). DNA methylation is maintained by DNMT1 during cell replication and has been related to the maintenance of epigenetic imprinting occurred during the fetal development, while DNMT3a and DNMT3b are *de novo* DNMTs (Lyko, 2018). DNA methylation recruits methyl CpG binding proteins (MeCP), blocking RNA polymerase activity by condensing chromatin and inhibiting the binding of transcription factors, which prevents the initiation of gene expression. DNMTs induce the demethylation of the CpG sites when inactive, promoting gene expression (Allis and Jenuwein, 2016). *Dnmt3a* and *Dnmt3b* are both localized in the spermatogonial cells in rat testes and *Dnmt3a* is also expressed in spermatocytes (Xu et al., 2015). Herein, we show that maternal diet influences slightly the gene expression of the epigenetic marker *Dnmt3a* in the testis of adult offspring. DNMT3a activity can play a role in adulthood, causing modifications on pre-existing epigenetic patterns (Lyko, 2018).

Evidence suggest that high-fat diet intake could alter the DNA methylation profile in the germline of adult male rats and that could partially transmit the altered epigenetic signatures of developmental importance *via* sperm to the embryo causing embryo loss (Deshpande et al., 2020). It is known that high-fat diet intake increases adiposity in the individual per se and several studies focusing on reproductive health have shown that either maternal or paternal high-fat diet intake is able to program increased adiposity in male offspring, besides increasing adiposity in the progenitors (Palmer et al., 2012; Fullston et al., 2013; Fan et al., 2018; Deshpande et al., 2020; Sertorio et al., 2021). Our model showed that high-fat and high-sugar diet intake increased the adipose tissue weight in both progenitors and that there was an interaction effect of maternal and paternal diet intake increased adiposity of retroperitoneal and epididymal adipose tissue in adult male offspring, evidencing the synergistic effect of the paternal and maternal diets combined.

Adipocyte hypertrophy has also been associated to an activated inflammatory response in adipose tissue, producing proinflammatory cytokines such as TNF-α, IL-1 and IL-6, and C-reactive protein (Segovia et al., 2014). Some studies have shown that the exposure of the fetus to inflammatory mediators in the uterus, provoked by maternal immune stimulation, produces an offspring that exhibits a proinflammatory phenotype. This inflammatory profile is activated due to changes in the developmental programming of the immune system (Mandal et al., 2013), increasing inflammatory responses in other tissues. Consistently, we observed increased adiposity in the female progenitor in gestation, which probably set a poor environment for offspring development, programming the pattern of inflammatory response in adult male offspring. In our study, maternal diet influenced the production of the proinflammatory cytokines TNF-α and IL-6 in the testis, demonstrating that an inflammatory response has been programmed. Nevertheless, an adequate and dynamic balance between pro- and anti-inflammatory mediators must exist and evolve over time to achieve control of the cause of inflammation without inducing tissue damage (Cicchese et al., 2018). IL-10 is an anti-inflammatory cytokine that plays a counter-regulatory role in the immune response to antigens, being produced in response to proinflammatory signals to prevent excessive inflammation (Rutz and Ouyang, 2016). Accordingly, the maternal diet also altered the IL-10 levels in our study, which may reflect a testicular adaptive response in the offspring.

The inflammatory response induced by the cytokines may generate ROS and higher levels of ROS could overload the tissue's antioxidant capacity. It could result in altered levels of the antioxidant enzymes SOD, CAT, and glutathione, and therefore, interfere with the production of testosterone, in the concentration of viable sperm and culminate in low sperm count (Azenabor et al., 2015; Tremellen, 2016; Fan et al., 2018; Yi et al., 2020). In our study, oxidative stress by maternal diet influence could be observed through the changed levels of SOD and CAT in the testis, that were altered along with the cytokines, and CAT and GST in epididymis. The oxidative imbalance did not translate into oxidative cell damage measured by this study, i.e., increased levels of MDA and CP. Another programming study reported cell damage by increased levels of MDA and nitrotyrosine in the testis and sperm when maternal exposure to a high-fat diet was prior to conception and the parameters assessed at different ages of older offspring (Rodríguez-González et al., 2015). The maternal influence and the interaction effect between both parents were able to decrease the number of spermatids in the testis, and consequently, the daily sperm production in the offspring. Other studies also showed the effects of maternal high-fat diet in male offspring related to oxidative imbalance in the testis and low daily sperm production (Sertorio et al., 2021). Impaired levels of SOD, CAT, and GPx were also observed in

testis and sperm (Rodríguez-González et al., 2015; Santos et al., 2015; Bautista et al., 2017).

Besides the inflammatory response caused by higher adiposity, Sertoli cells and germ cells in the seminiferous epithelium produce cytokines, including TNF-α, TGF family members, and interleukins (Lie et al., 2013). Germ cells undergo spontaneous degeneration during spermatogenesis in order to establish the size of the cell population. This degeneration occurs through the induction of apoptosis of these cells and the breaking of the tight junctions in Sertoli cells, as well as through the inhibition of steroidogenesis in Leydig cells (Lie et al., 2013). The increased concentration of proinflamatory cytokines could intensify this degeneration process, affect the function of cell junctions that forms the blood-testicular barrier, and impair sperm production (Siu et al., 2003; Lydka et al., 2012). It is possible that the influence in concentrations of the proinflammatory cytokines TNF-α and IL-6 have also increased the degenerative process of germ cells and/or the structural function of Sertoli cells, interfering with the production of sperm numbers and, therefore, with daily sperm production. Reame et al. (2014) showed that breastfeeding is a critical period and that maternal high-fat diet intake led to cell detachment in the seminiferous tubules of the offspring, leading to impairment in daily sperm production.

The increased adiposity caused by parental high-fat diet intake could still impact the hypothalamic-pituitary-gonadal axis of the male progeny, leading to hormonal disruption and predisposing the offspring to the development of central hypogonadism (Crean and Senior, 2019). High-fat intake results in the deregulation of adipokines, such as leptin, that plays an important role in reproductive health by stimulating gonadotropin-releasing hormone (GnRH) release (Tsatsanis et al., 2015). Testosterone is the main male steroid hormone and its production is dependent on the production of GnRH by the hypothalamus. GnRH activates in the pituitary gland the production of prolactin, LH and FSH, the hormones that regulate reproductive function.

LH activates the expression of StAR, that is responsible for cholesterol transport into the mitochondria in Leydig cells; the cholesterol side-chain cleavage enzyme (P450scc) then produces pregnenolone, which is converted into progesterone by enzymes of the smooth endoplasmic reticulum. Progesterone is converted into testosterone by 17β-HSD (Ross and Pawlina, 2011; Kierszenbaum and Tres, 2016). The expression of *Star* and *Hsd17b3* were not significantly altered in our study, and that could be due to the age of the animals or the type of diet that the offspring consumed during life. A study showed that male offspring 120 days-old from obese fathers and submitted to high-fat diet intake over life showed decreased gene expression of *StAR*, *P450scc*, and *17β-HSD* and consequently low circulating levels of testosterone when compared to male offspring from lean fathers (Sanchez-Garrido et al., 2018). Changes in serum testosterone levels due to paternal diet influence were observed in our study, which corroborates with several reproductive programming studies reporting the decrease of circulating levels of testosterone in male offspring of mothers or fathers fed an high-fat diet (Jacobs et al., 2014; Reame et al., 2014; Rodríguez-González et al., 2015; Navya and Yajurvedi, 2017; Mao et al., 2018; Sanchez-Garrido et al., 2018).

Another common feature observed in studies on reproductive programming is the lower sperm count in the cauda epididymis and vas deferens due to maternal or paternal high-fat diet intake (McPherson et al., 2014; Reame et al., 2014; Fullston et al., 2015; Rodríguez-González et al., 2015; Santos et al., 2015; Navya and Yajurvedi, 2017; Mao et al., 2018; Youngson et al., 2019). The spermatids produced by the testis pass through the epididymis, where the sperm acquisition of motility, the ability of sperm to fertilize, and the storage of sperm until ejaculation occurs. The exposure of these cells to a healthy epididymal microenvironment is crucial for maturation, which is also regulated by the transit time of sperm through the organ (Sertorio et al., 2021). Our results showed there were changed levels of CAT and in the production of the proinflammatory cytokine TNF-α in cauda epididymis due to maternal high-fat high-sugar intake. An interaction effect was observed in lower CP levels, an oxidative protein marker. Although maternal diet had changed the sperm number in caput/corpus epididymis, there were no changes in sperm transit time in this region. The changed sperm count in caput/corpus epididymis would probably be due to the low spermatid count in the testis. The changed CAT levels were probably able to fight the activation of the inflammatory response in cauda epididymis, influencing TNF-α and CP levels. Unbalanced levels of GST were observed due to paternal diet, and altogether, could have reflected the lack of change in sperm count and transit time in cauda epididymis, as well as maintaining the proportion of morphologically normal sperm from the vas deferens. In male offspring exposed to the maternal obesogenic environment at different stages of development, the epididymis transit time was also not altered (Reame et al., 2014).

Reproductive programming studies showed lower activity of the antioxidant enzymes, increased levels of stress markers, decreased number of normal sperm and sperm motility in the sperm of offspring of mothers submitted to high-fat diet intake (Rodríguez-González et al., 2015; Santos et al., 2015). Paternal high-fat diet intake was also shown to damage sperm parameters in offspring (Fullston et al., 2012; McPherson et al., 2014; Fullston et al., 2015). Although sperm count, transit time, and sperm morphology remained unchanged in our study, at a molecular level the cytokine production, activity of antioxidant enzymes and oxidative stress marker point out the interaction effect of maternal and paternal diet. According to our results, it is possible to suggest that the maternal effect is more pronounced than the paternal effect. That could be explained by the differences in paternal and maternal epigenetic inheritance, since maternal epigenetic reprogramming can occur at different temporal windows and alter more directly the offspring environment—the periconceptional environment, affecting early embryo reprogramming; the erasure and reacquisition of primordial germ cells during gestation; and the postnatal period (Radford, 2018). On the other hand, paternal epigenetic inheritance can be transmitted only through sperm, what could

reflect the less pronounced effect observed in the offspring phenotype.

5 CONCLUSION

Parental high-fat high-sugar diet intake prior to conception and/or during gestation and lactation could influence the reproductive health of male offspring that consumed control diet after weaning at a molecular level, showing the potential programming effects of isolated or combined intake, and that the maternal effect seems to be more pronounced than the paternal effect. High-fat high-sugar diet when consumed by the mother during gestation and lactation could affect the testicular epigenetic parameter, inflammatory response, oxidative balance, and daily sperm production of the offspring in early adulthood. The lower daily sperm production effect could be exacerbated by the interaction with the high-fat high-sugar diet consumed by the father prior to conception. Paternal diet influenced lower serum testosterone levels, which could have played a role in daily sperm production of the offspring. High-fat high-sugar diet intake by the parents have also altered the inflammatory response and antioxidant enzyme activity in the cauda epididymis. The alteration in the production of pro-inflammatory cytokine in the epididymis and the oxidative stress marker probably prevented deleterious effects in sperm at a morphologic level.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethic Committee on Animal Use of the Federal University of São Paulo (CEUA number 9856031018/UNIFESP).

AUTHOR CONTRIBUTIONS

MS, HC, and LP designed the research study. MS performed experimental procedures, analyzed the data and wrote the manuscript. HC and BC contributed with the statistical analysis. HC, ES, LM, AS, LS, and AJ performed experimental procedures. OA and DE provided laboratory support. LP contributed to the draft and reviewed the manuscript critically. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.867127/full#supplementary-material

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Sex-differential RXRα gene methylation effects on mRNA and protein expression in umbilical cord of the offspring rat exposed to maternal obesity

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Maternal obesity (MO) induces negative consequences in the offspring development. Adiposity phenotype is associated with maternal diet at early pregnancy and DNA methylation marks in the RXRα promotor at birth. Glucocorticoids play an important role in the regulation of metabolism through the activation of nuclear hormone receptors such as the RXRα protein. The aim of the study was to analyze steroid hormone changes at the end of pregnancy in the obese mother and RXRα gene methylation in the umbilical cord. For this purpose, in a well-established MO model, female Wistar rats were fed either standard chow (controls: C) or high-fat obesogenic diet (MO) before and during pregnancy to evaluate at 19 days of gestation (19 dG): 1) maternal concentration of circulating steroid hormones in MO and C groups, 2) maternal and fetal weights, 3) analysis of correlation between hormones concentration and maternal and fetal weights, 4) DNA methylation status of a single locus of RXRα gene near the early growth response (EGR-1) protein DNA binding site, and 5) RXRα mRNA and protein expressions in umbilical cords. Our results demonstrate that at 19 dG, MO body weight before and during pregnancy was higher than C; MO progesterone and corticosterone serum concentrations were higher and estradiol lower than C. There were not differences in fetal weight between male and female per group, therefore averaged data was used; MO fetal weight was lower than C. Positive correlations were found between progesterone and corticosterone with maternal weight, and estradiol with fetal weight, while negative correlation was observed between corticosterone and fetal weight. Additionally, male umbilical cords from MO were hypermethylated in RXRα gene compared to male C group, without differences in the female groups; mRNA and protein

expression of RXRα were decreased in F1 male but not in female MO compared to C. In conclusion, MO results in dysregulation of circulating steroid hormones of the obese mothers and low fetal weight in the F1, modifying DNA methylation of RXRα gene as well as RXRα mRNA and protein expression in the umbilical cord in a sex-dependent manner.

KEYWORDS

DNA methylation, sexual dimorphism, maternal diet, corticosterone, estradiol, steroid hormones, programming, fetal weight

Introduction

The developmental programming concept (Barker, 2002; Barker, 2005), also known as developmental origins of health and disease (DOHaD), states that challenges in critical developmental time windows alter development with persisten effects on offspring phenotype (McMullen and Swali, 2013). Obesity is the most prevalent nutritional disorder in childhood (Fryar et al., 2020; World Health Organization, 2020). Multifactorial mechanisms such as parental nutrition and lifestyle, psychosocial and neuroendocrine status, genetic background, physical activity, adverse intrauterine environment during pregnancy, lacking breastfeeding, among others, contribute to obesity in children (Lau et al., 2011; Parlee and MacDougald, 2014; Pauwels et al., 2019; Bautista et al., 2021).

During pregnancy, maternal nutritional status is a crucial factor for modulating developmental programming in the offspring (Lau et al., 2011). Experimental evidence using hypo and hypercaloric maternal diets in animal models directly correlates with epigenetic changes in fetal tissues and various effects on the offspring phenotypes (Ganu et al., 2012), generating a great debate on the specific weight of epigenetic effects in the etiology of obesity.

In human pregnancy, obesity can result in abnormal fetal growth (Vieira et al., 2017). One biological mechanism thought to underlie this relationship is the fetal epigenetic programming (Saffery and Novakovic, 2014) by circulating steroid hormones stimuli (Socha et al., 2016; Houshdaran et al., 2020). The fetus must adapt to the supply of nutrients crossing the placenta, where peripheral endocrine regulation is a determinant for maternal metabolism coming into play for adequate fetal growth (Murphy et al., 2006). The placenta produces numerous hormones, including progesterone and estrogens, that work together to regulate maternal metabolism and may have essential participation in the regulation of fetal size (Mucci et al., 2003). Moreover, this transitory organ also maintains the glucocorticoid balance between the mother and the fetus.

Maternal obesity during pregnancy is associated with a pro-inflammatory intrauterine environment and lipotoxicity in the placenta, which has a link with adverse long-term metabolic programming in the offspring (Saben et al., 2014). Early growth response-1 (EGR-1) protein induces inflammatory cytokines expression in the trophoblast (Saben et al., 2013). Interestingly, the DNA-binding element for early growth response (EGR) proteins in the promoter region of the retinoid X receptor alpha (RXRα) gene is susceptible to differential DNA methylation (Chávez-Lizárraga et al., 2018).

DNA methylation is an epigenetic mechanism involved in the interaction between nutritional status and modulation of gene expression in individuals (Choi and Friso, 2010). During early human pregnancy, maternal carbohydrate intake proportion is linked with changes of DNA methylation of the RXRα gene promoter in the umbilical cord at birth, which is correlated with adiposity in children by age 9 years (Godfrey et al., 2011). Moreover, a maternal diet enriched with essential nutrients for fetal development inversely correlates with RXRα methylation in the placenta, which is associated with the newborn anthropometric characteristics (Nakanishi et al., 2021). RXRα protein belongs to the steroid and thyroid hormone receptor superfamily, acting as a transcriptional factor of genes linked to developmental biology and adipocytokine signaling pathway (Zhang et al., 2015). These results suggest that maternal diet, including nutrients rich in methyl groups during pregnancy, affects DNA methylation of key genes as RXRα in placenta and umbilical cord, contributing to developmental programming of the offspring (Burdge et al., 2007; Harvey et al., 2014; Nakanishi et al., 2021).

Steroid hormones during pregnancy play a critical role in the regulation of metabolism through their interaction with intracellular receptors. This includes RXRα protein that acts as transcriptional factor causing changes on gene expression (Evans and Mangelsdorf, 2014). In rodents, maternal serum corticosterone is considered the main glucocorticoid involved in regulating the stress response, having significant repercussions on developmental programming with sexual dimorphism (Zambrano et al., 2015; Rodríguez-González et al., 2019). Therefore, steroid hormones, including glucocorticoids, may have crucial contributions to fetal programming *via* modulation of epigenetic changes. Further study of transcriptional regulation mechanisms could provide evidence of molecular mechanisms involved in obesity-induced cellular and tissue dysfunctions in energy expenditure metabolism and programming of the offspring.

The rat model used in this study is a well-characterized method for exploring maternal steroid hormones concentration and epigenetic changes in the offspring as causal mechanisms in

developmental programming influenced by maternal nutritional status (Zambrano et al., 2010; Rodríguez-González et al., 2015; Lomas-Soria et al., 2018). Epigenetic studies in the umbilical cord are important because, it is a tissue containing fetal vascular endothelial and mesenchymal stem cells with possible implications for future adiposity (Kadam et al., 2009). In addition, experimental evidence has been found that DNA methylation changes in the liver and heart induced by maternal diet are similar to those found in the umbilical cord (Lillycrop et al., 2005; Burdge et al., 2007).

In this study, we analyzed RXRα gene methylation (in the first exon near the response element of EGR-1) and mRNA and protein expression of RXRα in umbilical cords at 19 days of gestation offspring from obese rat mothers exposed to a high-fat diet. We examined maternal and fetal weights and their correlation, considering the low fetal weight in rodents as an adverse perinatal outcome associated with maternal obesity. Therefore, we hypothesized that obesity during pregnancy induces dysregulation in circulating steroid hormones of the obese mothers, disturbing DNA methylation of the RXRα gene and expression of RXRα mRNA and RXRα protein in umbilical cord of the offspring in a sex-dependent manner.

Materials and methods

Maternal obesity rat model

Experiments were conducted by following the principles of the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (INCMNSZ), Mexico City, Mexico (CINVA, institutional reference numbers: BRE-1868-16/19-1) and performed according to the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (http://www.nal.usda.gov/awic/animal-welfare-act). Female albino Wistar rats were born and maintained in the INCMNSZ animal facility, accredited by and adhering to the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) standards.

For details in general procedures relating to the care of rats, maintenance, maternal diet, mating process, breeding, and management of control and obese mothers, consult the following references (Zambrano et al., 2010; Zambrano et al., 2021; Rodríguez-González et al., 2019). Briefly, at delivery on day 0, litters that provided founder generation (F0) mothers were culled to 10 pups, each containing at least four females. At weaning (day 21) one female F0 pup from each litter was randomly assigned to either a maternal control (C) group fed laboratory chow (LabRodent Diet 5001, Fort Worth, TX, United States that contains 23.9% protein, 5.0% fat, 31.9% polysaccharide, 23.2% simple sugars, 5.0% fiber, 7.0% minerals and ~1.0% vitamins (w/w); energy provided = 3.4 kcal/g) or to a maternal obesity group (MO) fed a high energy, obesogenic diet (containing 22.5% protein, 20.0% animal lard, 5.0% fat, 20.5% polysaccharide, 20.5% simple sugars, 5.0% fibre, 5.0% mineral mix, 1.0% vitamin mix, (w/w); energy provided = 4.9 kcal g^{-1} (Rodríguez-González et al., 2019). There were not differences in F0 body weight at the initial of the study (21 d) among C and MO groups. The high-energy obesogenic diet was produced in the specialized dietary facility of the INCMNSZ. Thus, each F0 group had only one female from any litter, and F0 females in different groups, but not within groups were sisters, providing homogeneity in F0 mothers' developmental programming and genetics. We report data with an $n = 6$ per F0 group.

Body weight was measured weekly from weaning to 120 postnatal days (PND) in F0 MO and C groups. F0 young adult female rats were placed with proven male breeders following 120 PND and conceived during the next estrous cycle. Female rats were mated overnight with proven male breeders (Zambrano et al., 2020). The day on which spermatozoa were detected in a vaginal smear was designated as conception day 0. Fertility rate for C group was 75% and for MO group 25%. Throughout pregnancy, body weight (g) was recorded daily in both F0 groups until 19 dG, knowing that the average gestation time in the rat is 21 days (Figure 1).

Steroid hormones quantification

At 19 dG, F0 rats were euthanized by decapitation with anesthesia, and blood samples took from the neck and were centrifuged at 3,500 rpm for 15 min at 4°C to remove red blood cells, and the serum was stored at −20°C until all samples were analyzed. Enzyme-linked immunosorbent assays (ELISAs) were performed, measuring samples by duplicate to quantified concentrations of serum hormones following manufacturer's instructions for commercial rat kits. Estradiol (pg/ml) and corticosterone (ng/ml) concentrations were quantified using ELISA kits from DRG International, Inc. (EIA-4399 and EIA-5186, respectively; Springfield, NJ 07081 United States) and progesterone (ng/ml) and testosterone (ng/ml) concentrations were quantified using the kits from SIEMENS Immulite/Immulite 1000 Systems (LKPW1 and LKTW1, respectively; Llanberis, Gwynedd, LL55 4EL United Kingdom).

Offspring litter measurements and tissue collection

At 19 dG, litter size, placenta and fetus (F1) weights as well as litter mass (total fetal and placenta weight) were recorded. The gonads of the fetus were observed with the support of a stereoscopic microscope by personnel trained to validate the

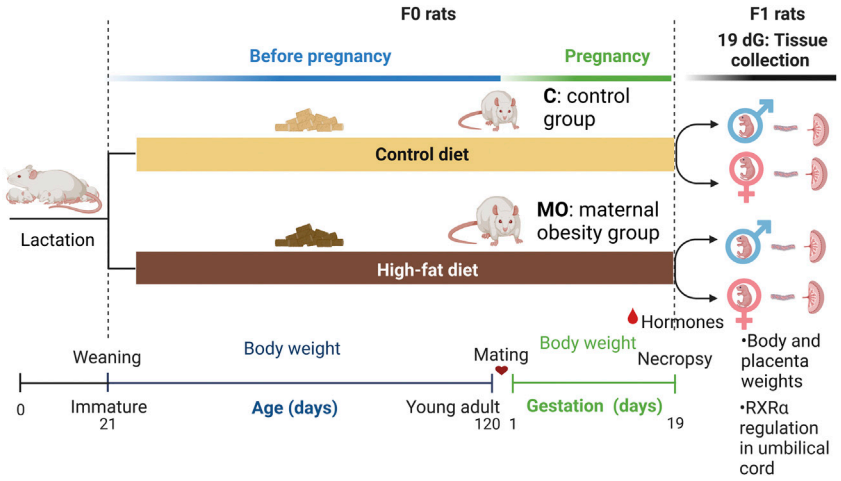

FIGURE 1
Maternal obesity study design. Experimental groups (n = 6 per maternal diet group), Female (F0) rats were fed with control (C) or high-fat (MO) diet before and during pregnancy and lactation. Mating around 120 days of age; dG, days of gestation; RXRα, retinoid X receptor alpha.

sex and sex ratio per litter. Umbilical cord samples from the fetuses of each litter were collected in pools divided by sex and maternal diet group, preserving them in RNAlater (Invitrogen) following instructions of manufacture until their use.

DNA and RNA extractions

Twenty-five milligrams of umbilical cord pooled from each litter were weighed and incubated in enzymatic tissue digestion solution [50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 1% SDS pH 8.0, and 0.5 mg/ml of proteinase K (Invitrogen)] at 50°C for 3 h, and then homogenized. DNA and RNA extraction of tissue samples was performed using 500 µl/sample of TRIzol reagent (Invitrogen) following manufacturers' instructions. The nucleic acid quality was assessed by measuring the absorbance at 260/280 nm and was quantified with a Thermo Scientific Nanodrop 2000c Spectrophotometer (Thermo Scientific). The integrity of DNA and RNA was verified by 1% agarose gel electrophoresis and ethidium bromide staining. Isolated DNA and RNA samples were preserved at −76°C until examination.

Primer designs and MS-HRM assay

Gene sequence of RXRα was obtained from the UCSC Genome Browser database on Rat July 2014 (RGSC 6.0/rn6) assembly. This location in the chr3:6,211,867-6,211,974 (107 pb) from the CpG 169 island corresponds to the first exon of the gene containing the response element [GCG(G/T)GGGCG (Lee, 2014)] for EGR-1 binding site in the rat genome. According to the MS-HRM technique conditions (Wojdacz and Dobrovic, 2007) the primers designed are the following: RXRα-F, 5′- GGG CGA GGG AGG GGG T -3′ and RXRα-R, 5′- CTA ACT CTC GAT ACC GCC AC-3′.

Five hundred milligrams of DNA were treated using the EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's protocol. The recovered bisulfite-treated DNA was quantified in the spectrophotometer using the absorption coefficient at 260 nm.

MS-HRM conditions for the site-specific analysis of the RXRα gene were as follows: 1) for amplification, 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 10 s at the primer annealing temperature (54°C), and 15 s at 72°C; 2) for high-resolution melting analysis, 1 min at 95°C, 5 s at 72°C, and continuous increase to 95°C with 50 acquisitions/°C; and 3), a cooling setting of 30 s at 40°C. MS-HRM assays were performed three times in duplicate white 96-well plates using a LightCycler 480 Instrument (Roche).

Direct Sanger sequencing

Direct sequencing was performed in at least 10% of MS-HRM products in both alleles to validate RXRα methylation results. Previously, 500 ng of universal rat genomic DNA standards [high methylated control (HM) and low methylated control (LM) (EpigeneDx)] were treated using the EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's protocol. 50 ng of treated DNA standards and MS-HRM products were re-amplified

with the RXRα primers described above using a BigDye Terminator Kit and sequenced with an ABI prism 370 DNA sequencer (Applied Biosystems). The nucleotide sequences were aligned using Mega 6.06 and manually adjusted in the text editor. Initial identification of the sequences was made after performing BLAST searches of the NCBI database. The electropherogram quality was visualized with BioEdit Sequence Alignment Editor software v7.2.5, and CpG highlighting was simplified using the BiQ Analyzer software tool (Chávez-Lizárraga et al., 2018).

Analysis of gene expression by RT-qPCR

Five micrograms of total RNA from umbilical cord samples were synthesized to cDNA using the Transcriptor First-Strand cDNA synthesis kit (Roche) following the manufacturer's instructions. Assay efficiency was calculated using a dynamic range of cDNA dilution series (1:1, 1:2, 1:10, 1:100, 1:1,000, and 1:10,000). Quantitative real-time PCR was performed using the LightCycler TaqMan Master Kit (Roche), and TaqMan gene expression assay probes for RXRα and β-actin were purchased from Thermo Fisher Scientific. qPCR conditions for RXRα gene expression were the following: for amplification, 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 30 s at the primer annealing temperature (60°C), and 10 s at 72°C. qPCR assays were performed three times in duplicate using a LightCycler Nano Instrument (Roche). Gene expression data were normalized using β-actin expression. Fold change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Protein extraction and Western blotting

Fifty mg of umbilical cord sample pools from litters per sex were pre-treated with lysis buffer [50 mM Tris–HCl, 1% Nonidet P-40, protease inhibitor cocktail, pH 7.4 (Sigma Aldrich)] at 37°C for 24 h. The next day, samples were incubated in ice-cold lysis buffer for 1 h and homogenized. Homogenized samples were centrifuged at 13,000 rpm at 4°C for 5 min, and the supernatant was obtained and protein quantified by Bradford assay (Bio-Rad). Extracts were preserved at −76°C until their examination.

Fifty micrograms of protein in Laemmli buffer (1:1) were loaded in 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Thermo Scientific Pierce). The membrane was blocked with 5% non-fat dry milk dissolved in TBST for 30 min at room temperature. Blots were incubated with rabbit anti-RXRα [1:2,000 (Abcam)] or rabbit anti-β actin [1:2,000 (Abcam)] overnight at 4°C, washed with TBST, and incubated with secondary goat anti-rabbit- HRP [1:5,000 (Abcam)] for 2 h at room temperature. Image acquisition and densitometry analysis were performed using Image Lab software version 5.2.1 build 11 (Bio-Rad).

Statistical analysis

Normality test was performed by Kolmogorov-Smirnov. Data are expressed by mean ± standard deviation (SD) for parametric values, while median and interquartile range (IQR) for non-parametric. $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered statistically different. The comparison of litter size, sex ratio, mother, fetuses and placenta weights, and steroid hormones concentration between MO and C groups by Welch's *t*-test. Pearson's test was used to analyze correlations among maternal, fetal and placental weights, and hormones concentration. Since there were no differences in fetal weight between male and female per group, we have done correlations using averaged data.

The resulting melting curves after MS-HRM assays were converted to negative derivate peaks, for which the negative derivative of the fluorescence over the derivative of temperature (−dF/dT) is plotted against the increasing temperature. Heterogeneous methylation can only be estimated in a qualitative manner, for which the melting peaks in the highest melting temperature mean high methylation while melting peaks in the lowest temperature mean low methylation (Hussmann et al., 2018). The number of methylated CpG dinucleotide (mCpG) was accounted for directly Sanger sequencing results in both alleles (forward: F and reverse: R), and the average of CpGs between both alleles was determined.

RXRα mRNA and protein expression in umbilical cord were compared by nested *t*-test and two-way ANOVA. Analysis of correlation between RXRα mRNA and protein in umbilicar cord were performed using Spearman's rank correlation test (r). Analysis and plots were performed using GraphPad Prism (version 9.2.0).

Results

Maternal, fetal, and placenta weights

The MO group had similar body weight since 21 d (C = 51.4 ± 0.9 and MO = 51.7 ± 0.9 g, $p > 0.05$) until 63 d (C = 231 ± 3.1 and MO = 264 ± 5.5 g, $p < 0.05$), in which MO had an increased body weight compared to C until the end of the experiment (Figures 2A–C). During gestation, cumulative and total weight gain (C = 124 ± 12.5 and MO = 112 ± 20 g, $p > 0.05$) were similar between C and MO groups. Using averaged data, during gestation food intake per day was lower in MO than C group (C = 26.2 ± 3.5 and MO = 21.5 ± 1.8 g/day, $p <$

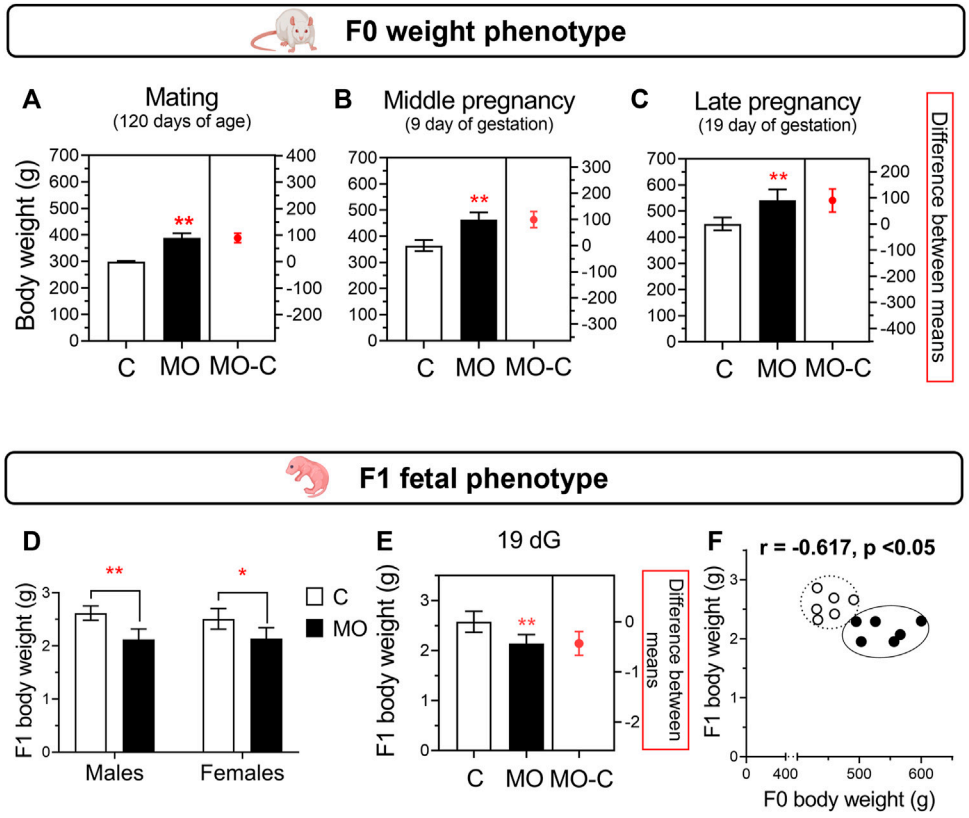

FIGURE 2
Maternal and fetal weight phenotypes. F0 body weight at mating **(A)**, middle pregnancy **(B)**, and late pregnancy **(C)**. Fetal body weight **(D)** and sex-averaged fetal weight **(E)** at 19 dG. Correlation between maternal and sex-averaged fetal weights **(F)**. Mean ± SD in bar plots (n = 6/group); $*p < 0.05$ and $**p < 0.01$ vs. C.

0.05) but energy intake was higher in MO than C (C = 89.2 ± 11.8 and MO = 103.2 ± 8.8 Kcal/day, $p < 0.05$). The energy intake per gram of body weight gained was similar between C and MO (C = 4,311 ± 2,597 and MO = 4,790 ± 2,772 Kcal/g, $p > 0.05$).

Litter size were similar between C and MO (C = 14.5 ± 1.8 and MO = 14.2 ± 4.0 pups/litter; $p > 0.05$). There were no differences in male:female ratio (C = 1.1 and MO = 1.0; $p > 0.05$). At 19 dG, placenta and body weight were similar between male and female for both groups. There were no differences between C and MO placenta weight (C = 0.62 ± 0.07 and MO 0.55 ± 0.11 g; $p > 0.05$). Total fetal weight in the litter was higher in C vs. MO (C = 37.4 ± 5.8 and MO = 29.7 ± 6.1 g, $p < 0.05$; Figures 2D,E); total placenta weight in the litter was similar between C and MO (C = 6.37 ± 3.8 and MO = 7.4 ± 1.0 g, $p = 0.052$). Total mass (fetal and placenta) per litter were similar among groups (C = 46.7 ± 7.5 and MO = 37.5 ± 7.0 g, $p = 0.053$). There is a negative correlation between maternal and fetal weight (Figure 2F), but no correlation between maternal and placental weight ($r = -0.115$, $p > 0.05$).

Maternal serum steroid hormones and their correlation with maternal and fetal weights

Progesterone and corticosterone maternal serum concentrations were higher, and estradiol lower in MO group compared to C group. Testosterone maternal serum concentrations were similar between C and MO groups (Figures 3A–D). Positive correlations were found between progesterone and corticosterone with maternal weight, and estradiol with fetal weight, while negative correlation were found between corticosterone and fetal weight. No correlation were found between testosterone and maternal and fetal weights (Figures 3E–L).

RXRα gene methylation status near early growth response-1 binding site

In the locus analyzed (Figure 4A), we observed an increased heterogeneous methylation state of the RXRα gene in the

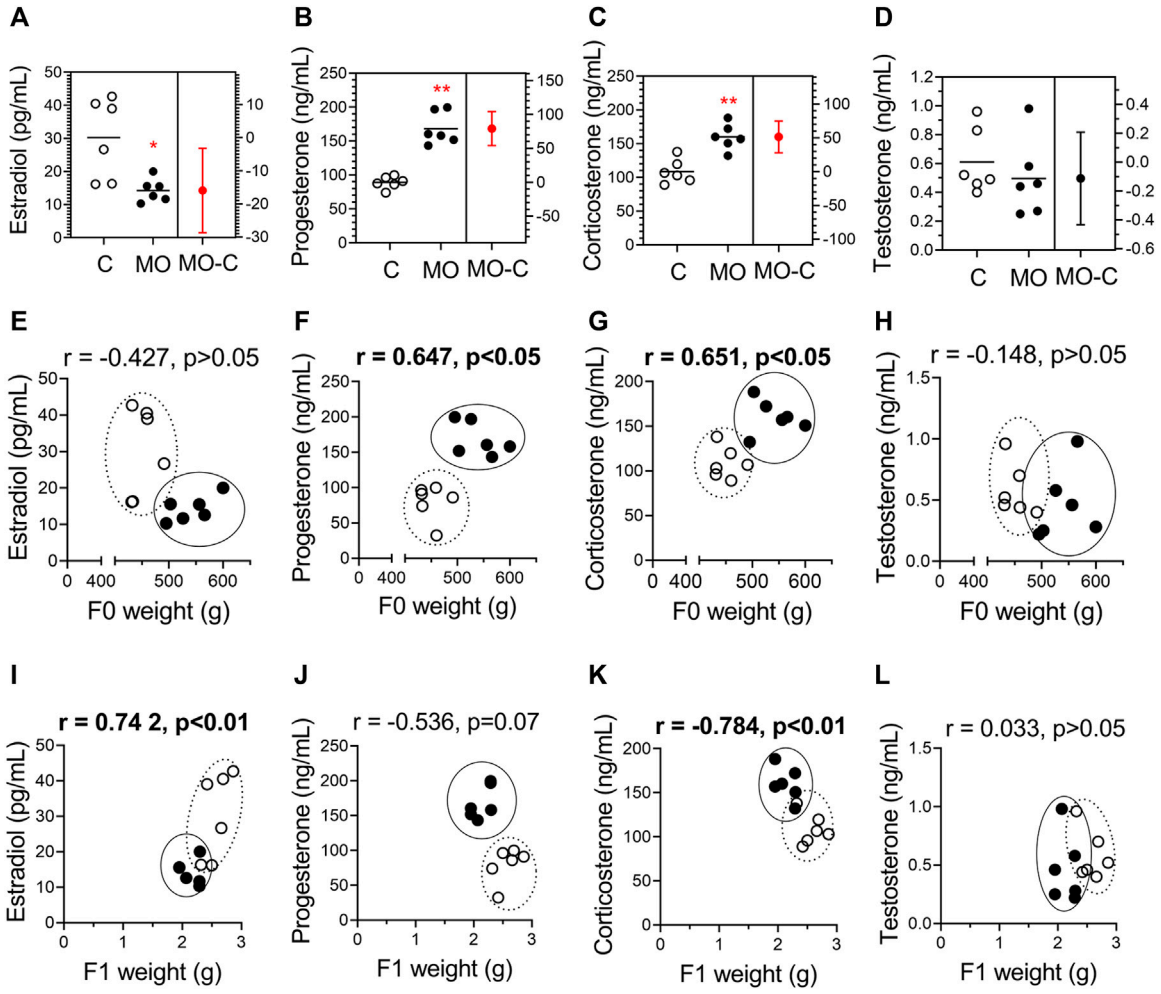

FIGURE 3
Stereoid hormones and their correlation with maternal and fetal weights. Maternal serum concentrations of estradiol **(A)**, progesterone **(B)**, corticosterone **(C)**, and testosterone **(D)** at 19 dG. Mean ± SD (*n* = 6/group); **p* < 0.05 and ***p* < 0.01 vs. **(C)**. Pearson correlations (r) between hormones and maternal weight **(E–H)** and fetal weight **(I-L)**; *C* = white circles, MO = black circles.

umbilical cord of MO fetuses in comparison to their controls. Furthermore, DNA methylation status of the RXRα gene in the umbilical cord of male MO fetuses was higher than in female MO fetuses (Figure 4B).

Validation of RXRα gene methylation status

MS-HRM products were sequenced for identifying specific sites of mCpGs between forward and reverse alleles in the examined sequence and to validate previous results. Our findings corroborated a high state of heterogeneous methylation composed of a range of 61%–78% mCpGs between alleles in the cord RXRα gene of male MO fetuses, while in female MO fetuses, the high state of heterogeneous methylation was composed of 28%–56% mCpGs between alleles. Respective controls of male and female fetuses showed a state of umbilical cord RXRα gene methylation about 17%–28% of mCpGs (Figure 4C). Finally, the total of mCpGs between alleles was higher in male MO fetuses than male C fetuses, and no differences were found between female MO and C fetuses (Figures 5A,B).

Expression of RXRα mRNA and RXRα protein

At 19dG, RXRα mRNA and protein expression in the male MO umbilical cord was lower compared to C; no differences were

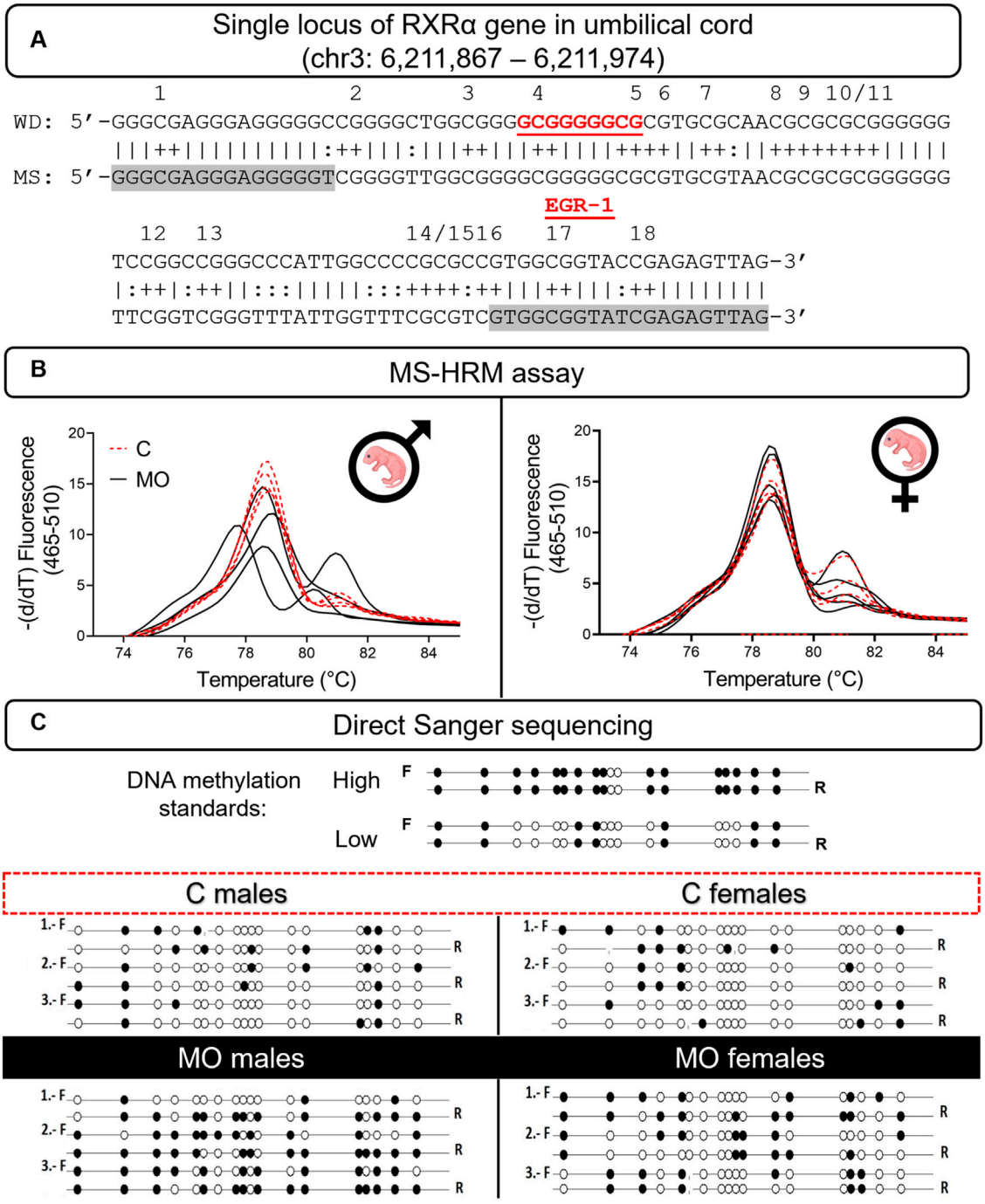

FIGURE 4
DNA methylation status of RXRα gene in offspring's umbilical cord. RXRα sequence in a single locus of the chromosome 3 in the rat genome. DNA-binding site of EGR-1 transcriptional factor (red letters); primers sequence (highlighted in grey); CpG sites—numbers over sequence **(A)**. Melting peaks plots (*n* = 6/group/sex) show heterogeneuously methylation **(B)**. Lollipop diagram shows the validation of heterogeneuously methylation and individual mCpGs **(C)**. C, control; F, forward sequence; mCpGs, methylated CpG dinucleotides; MS, methylated sequence; MO, maternal obesity; R, reverse sequence; WD, wild sequence.

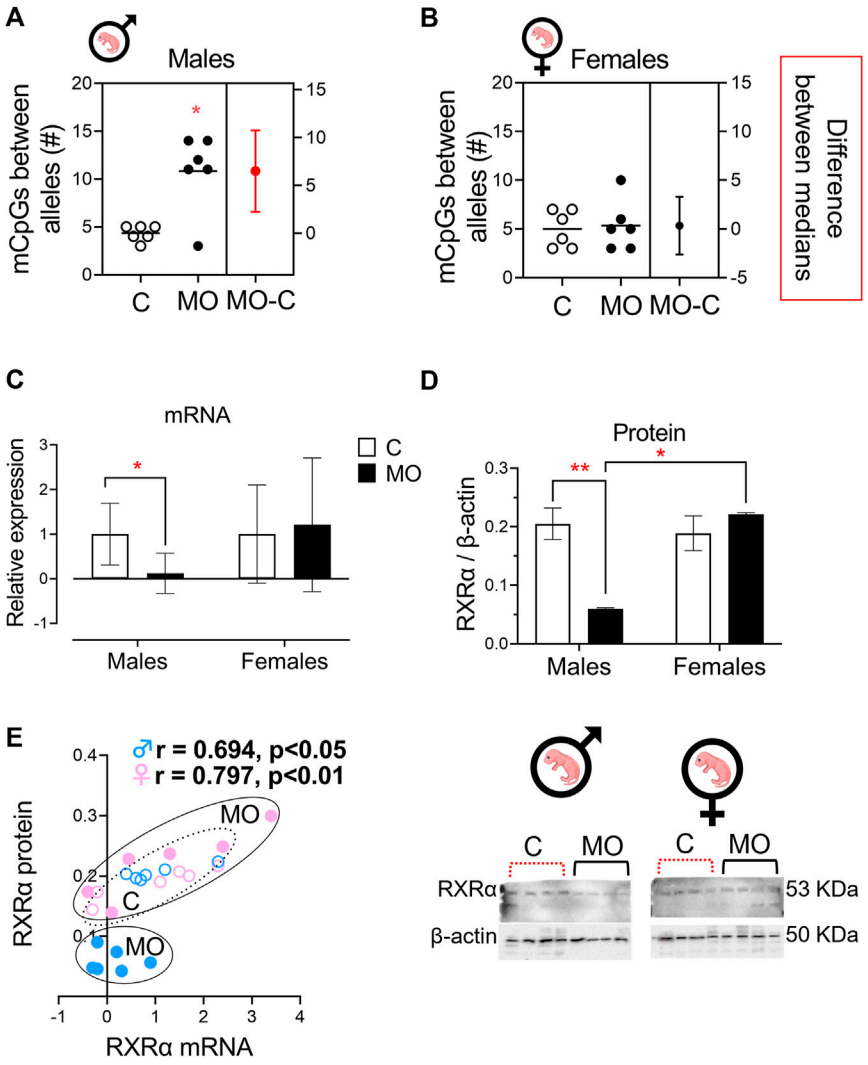

FIGURE 5
Allele-specific methylation and effects on RXRα mRNA and protein expression. Median with IQR. The average number of mCpGs in each allele (n = 3/group/sex) in umbilical cord of males **(A)** and females **(B)** offspring. Relative RXRα mRNA **(C)** and RXRα protein expression **(D)** (n = 6/group/sex). A representative western blot is showed below RXRα protein plot (n = 4/group/sex). Spearman correlation between RXRα mRNA and protein expression **(E)**. *p < 0.05 and **p < 0.001 vs. C.

found in the female groups (Figures 5C,D). There is a positive correlation between RXRα relative mRNA and protein expression for both male and female groups (Figure 5E).

Discussion

Maternal obesity induces epigenetic changes in the offspring from fetal life onward that lead to metabolic problems including obesity in adulthood (Parlee and MacDougald, 2014; Rodríguez-González et al., 2019). Most of the experiments to study developmental programming due to maternal obesity and western diets have been conducted in rodents at different periods of maternal exposure to obesogenic diets and offspring developmental windows (pregnancy and/or lactation) (Bellisario et al., 2015; Rodríguez-González et al., 2015). It is important to consider the precise dietary components (macro- and micronutrients), food intake and extent and duration of MO and maternal high fat or sugar (or both) dietary intake before conception and during gestation. In this study we used the pregnant obese rat fed with high fat diet before and during pregnancy as experimental animal model to study maternal steroid hormones concentration, RXRα gene methylation near the EGR-1 binding site in the umbilical cord, as well as placenta and fetal weight at the end of gestation (19 dG).

Steroid hormones play an important role during pregnancy in developmental programming. In humans and rodents, maternal obesity during pregnancy has been associated with alterations of progesterone, estradiol, testosterone, and glucocorticoids (Rodriguez -Gonzailez et al., 2015; Vega et al., 2015; Maliqueo et al., 2017; Rodríguez-González et al., 2019). Circulating adrenal steroid hormones are small lipophilic molecules that regulate gene expression in a great variety of tissues, including the uterus, endometrium, and umbilical cord (Travers et al., 2018). These molecules bind to cognate receptors that exert post-translational modifications through interactions and nuclear translocations with cell and tissue-specific co-regulators (Houshdaran et al., 2020). Adrenal steroid hormones exert most of their physiological and regulatory functions of metabolic homeostasis through the binding and activation of orphan nuclear hormone receptors (Evans and Mangelsdorf, 2014), as is the case of RXRα protein.

Here, we investigated the maternal serum concentration of steroid hormones of F0 at 19 dG, resulting in increased progesterone and corticosterone and a decreased estradiol in the MO group compared to C, without changes in testosterone. The increased concentration of progesterone and the decreased concentration of estradiol in F0 MO group could be associated with the control of maternal body weight homeostasis to accommodate the fat deposition required to support fetal development and lactation, as well as deleterious maternal and placental functions (Zambrano et al., 2005; Lappas et al., 2020). The increased concentration of corticosterone in F0 MO group is consistent with our previous data before gestation and at the end of lactation, confirming that high serum concentrations of this glucocorticoid are a common featuring of response to stress generated by maternal obesity that repercusses on developmental programming (Zambrano et al., 2015; Rodríguez-González et al., 2019). One study in mice showed the increase of corticosterone concentration in obese mothers, maternal stressful challenge during pregnancy by high-fat diet comsumption, decreased placenta activity of 11beta-dehydrogenase-2 which implies that the protection to prevent the transplacental passage of surplus corticosterone is diminished, some of the consequences were the impairing neuroendocrine system and neural activity in the offspring (Bellisario et al., 2015). Although the adrenal gland is responsible for corticosterone production in the rat, the enzyme 11 beta-hydro steroid dehydrogenase—1 reductase in the adipose tissue can biotransform dehydrocorticosterone into corticosterone (Tomlinson et al., 2004) whereby adipose tissue can increase corticosterone concentration in the obese mother and be in part responsible of the accumulation of adipose tissue in the offspring in adult life.

DNA methylation is an epigenetic mechanism resulting in modulation of gene expression, mainly mediated by the control of transcription factors binding sites. Among other genes, specific DNA methylation marks have been identified in the RXRα gene promoter in newborns, and it has been associated with later adiposity in school-age children (Godfrey et al., 2011). Previous work in humans in our lab showed variability in DNA methylation of the RXRα gene promoter containing elements of response for EGR proteins in newborn's cord blood, indicating the presence of diverse expression of this gene (Chávez-Lizárraga et al., 2018). Using the rat model, we confirmed the presence of heterogeneous RXRα gene methylation near the EGR-1 binding site in the fetus's umbilical cord, showing that male fetal offspring exposed to maternal obesity had hypermethylation in the RXRα gene near the EGR-1 binding site. Experimental evidence supports that EGR-1 protein is a mediator for lipotoxicity-induced cytokine gene expression in the placentas from pregnant women with obesity, suggesting a relevant contribution to the effects of obesity on fetal programming (Saben et al., 2013).

A single locus of RXRα gene promoter methylation in umbilical cord has been associated with fat phenotypes of the offspring during childhood (Godfrey et al., 2011; Harvey et al., 2014). Moreover, experimental evidence in the Tet1 knockout

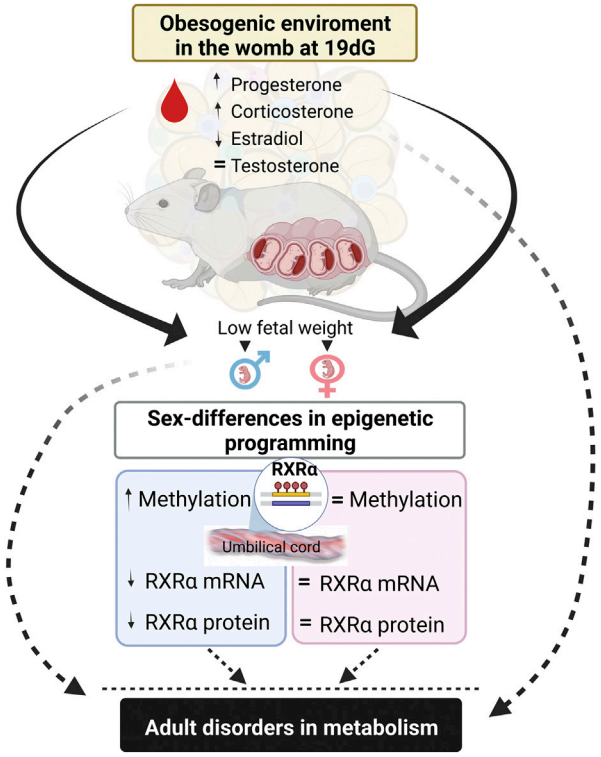

FIGURE 6
Summary of findings. Maternal high-fat diet modify stereoid hormone concentrations that correlates with low fetal weight and promotes umbilical cord hypermethylation in RXRα gene and decrases mRNA and protein expressions in a sex-dependent manner. These findings explain potential mechanism of offspring metabolic programming by maternal obesity.

mice showed that RXRα gene expression and RXRα protein activity are crucial for adipogenesis and adipocyte differentiation through DNA demethylation (Qian et al., 2021), showing that RXRα plays a critical role in adipogenesis. Previously, we have shown in rat offspring from obese mothers, fat tissue expansion (Ibáñez et al., 2018) and premature metabolism aging (Rodríguez-González et al., 2019) in a sex dependent manner.

Our data in umbilical cord are in accordance with other studies that showed DNA methylation of the RXRα gene as one of the main epigenetic mechanisms implied in the regulation of RXRα mRNA and RXRα protein expressions in fetal organs/cells as the placenta and trophoblasts (Pospechova et al., 2009; Nakanishi et al., 2021). DNA methylation of RXRα in the placenta correlates inversely to RXRα mRNA expression linked to maternal choline consumption, birthweight and body mass index in humans (Nakanishi et al., 2021). Our evidence of distinct RXRα mRNA and protein expressions between umbilical cords of male and female fetuses exposed to high-fat diets supports the hypothesis about a sex-specific response based on RXRα regulation linked to lipid metabolism in rodent tissues (Kosters et al., 2013) and the endocrine system (Sarr et al., 2012).

Findings discussed here thus far support the idea that maternal obesity and high-fat diet during pregnancy contributes to the epigenetic programming and explains a potential mechanism to develop lifelong metabolic problems in the offspring. Further exploration of mechanisms involved in the sexual dimorphism of metabolic imprinting is needed to propose targeted interventions that have an impact on the offspring life quality.

In summary, maternal obesity increases corticosterone and decreased estradiol serum levels at the end of gestation, which correlates with lower fetal weight. Interestingly, we observed male umbilical cord hypermethylation of the RXRα gene, with no changes in females, showing sexual dimorphism in fetal stages. These findings help to explain potential mechanisms of maternal obesity in the metabolic programming of the offspring (Figure 6).

In conclusion, serum steroid hormones changes in the obese mothers negative impacts fetal weight; obesogenic environment induces epigenetic changes of RXRα methylation in umbilical cord in a sex-specific manner. These findings show a potential mechanism explaining the association between maternal obesity with adipose tissue dysregulation, impaired metabolism and lifelong obesity in the offspring.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (INCMNSZ), Mexico City, Mexico (CINVA, institutional reference numbers: BRE-1868-16/19-1).

Author contributions

Authors agree to be accountable for all aspects of the work and read and approved the final review manuscript. EC-S, LR-C, and EZ: design of the work and drafting it; IL-T, LV-H, LR-C, GLR-G, RC, PZ-S, and AD-L: acquisition of data; EC-S, IL-T, and LR-C: analysis of data; EC-S, LR-C, FV-O, and EZ interpretation of data; EZ: conception of the animal model; FV-O and EZ: financial support.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptome and morphological analysis on the heart in gestational protein-restricted aging male rat offspring

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Background: Adverse factors that influence embryo/fetal development are correlated with increased risk of cardiovascular disease (CVD), type-2 diabetes, arterial hypertension, obesity, insulin resistance, impaired kidney development, psychiatric disorders, and enhanced susceptibility to oxidative stress and inflammatory processes in adulthood. Human and experimental studies have demonstrated a reciprocal relationship between birthweight and cardiovascular diseases, implying intrauterine adverse events in the onset of these abnormalities. In this way, it is plausible that confirmed functional and morphological heart changes caused by gestational protein restriction could be related to epigenetic effects anticipating cardiovascular disorders and reducing the survival time of these animals.

Methods: Wistar rats were divided into two groups according to the protein diet content offered during the pregnancy: a normal protein diet (NP, 17%) or a Low-protein diet (LP, 6%). The arterial pressure was measured, and the cardiac mass, cardiomyocytes area, gene expression, collagen content, and immunostaining of proteins were performed in the cardiac tissue of male 62-weeks old NP compared to LP offspring.

Results: In the current study, we showed a low birthweight followed by catch-up growth phenomena associated with high blood pressure development, increased heart collagen content, and cardiomyocyte area in 62-week-old LP offspring. mRNA sequencing analysis identified changes in the expression level of 137 genes, considering genes with a p-value < 0.05. No gene was. Significantly changed according to the adj-p-value. After gene-to-gene biological evaluation and relevance, the study demonstrated significant differences in genes linked to inflammatory activity, oxidative stress, apoptosis process, autophagy, hypertrophy, and fibrosis pathways resulting in heart function disorders.

Conclusion: The present study suggests that gestational protein restriction leads to early cardiac diseases in the LP progeny. It is hypothesized that heart dysfunction is associated with fibrosis, myocyte hypertrophy, and multiple abnormal gene expression. Considering the above findings, it may suppose a close link between maternal protein restriction, specific gene expression, and progressive heart failure.

KEYWORDS

fetal programming, gestational protein-restriction, cardiovascular disease, arterial hypertension, myocyte hypertrophy, heart miRNA transcriptome

Introduction

The developmental origin and mechanisms related to cardiovascular disease (CVD) are reasonably well known in human and animal models. According to the World Health Organization (World Health Organization, 2021), in humans, CVD is the leading cause of death globally, reaching 32% (17.9 million) worldwide deaths in 2019. The risk factors were generally associated with maternal intrauterine environment commitment. Barker et al. (1989) were the first to observe the inverse relationship between birth weight and CVD prevalence, interpreting the embryonic and fetal environments as a new component in the etiology of these diseases (Barker et al., 1989b). Epidemiological and experimental studies have demonstrated the intimate association between perinatal food restriction and a higher prevalence of CVD in adulthood [Langley-EvansGardner and Welham, 1998; Roseboom et al., 2000; Hinton et al., 2018; Assalin et al., 2019; Mariano et al., 2021]. From Barker's hypothesis, Alan Lucas grounded fetal programming in 1991 (Barker et al., 1989a; Lucas, 1991). Fetal programming could be caused by psychological and nutritional stress, as well as by placental ischemia. During experimental studies of fetal/embryonic development stages, these phenomena lead to long-term adverse effects on organs structure and function with an increasing predictive chance of developing chronic disorders (Lucas, 1991; Mesquita et al., 2010a,b; Sene et al., 2013; Sene et al., 2018; Sene et al., 2021; Lamana et al., 2021), including increased risk of cardiovascular and metabolic diseases (Langley-Evans et al., 1999; Gluckman & Hanson, 2004; McMullen et al., 2005; Burdge et al., 2007; Hanson & Gluckman, 2008) in adult life. Studies in rodents have emphasized that fetal programming results in low birth weight, which is associated with an increased risk of arterial hypertension, cardiovascular disorders, and chronic renal failure in adulthood (Mesquita et al., 2010a,b; Sene et al., 2013, 2018, 2021; Lamana et al., 2021; Mariano et al., 2021; Grigoletti-Lima et al., 2022). In response to a hostile intrauterine environment, the fetus/embryo undergoes adaptations that accelerate the maturation and differentiation of tissues and organs to the detriment of cell proliferation, restricting fetus growth (Fall 2013). The mechanisms by which fetal-programmed offspring lead to a higher incidence of CVD are not fully understood. However, cardiovascular disorders probably result from progressive epigenetic events that lead to an imbalance in the development and maintenance of the cardiovascular system (Barker et al., 1989b; Lakatta, 2003; Odden et al., 2011). Prior study has suggested that heart dysfunction is also associated with enhanced heart fibrosis and myocyte hypertrophy associated with multiple miRNA expression changes (Assalin et al., 2019). In addition, the gestational commitment overlaps with age-associated factors (smoking, physical inactivity, changes in diet, and lipid metabolism), emphasizing the importance of interaction to the increased CVD incidence over the years (Gluckman & Hanson, 2004; Hu et al., 2004; McMullen et al., 2005). Therefore, prior research assumes that the LP offspring induced a precocious aging process that culminates in premature animal death (Grigoletti-Lima et al., 2022). So, it is plausible that gestational protein restriction and its effects may influence gene expression and predispose cardiovascular disorders, which in turn may impact and modify the survival curve of the LP progeny compared to NP offspring. Considering the above findings, this study evaluated a close link between maternal protein restriction, specific gene expression, progressive heart failure, and reduced survival time of these animals. Thus, we may suppose that the current study may contribute to the best understanding of the onset of cardiovascular disease in adult offspring from the mother submitted to adverse events during pregnancy.

Material and methods

Animals and experimental design

The experiments were conducted on male offspring originating from inbred female and male Wistar HanUnib rats (250–300 g), sibling-mated, from the Multidisciplinary Center for Biological Research in Science in Laboratory Animals—Unicamp (CEMIB) with free access to water and standard rodent chow (Nuvital, Curitiba, PR, Brazil). The general guidelines established by the Brazilian Council for Animal Experimentation (CONCEA) were followed throughout the investigation. The Institutional

Ethics Committee reviewed and approved the experimental protocol (CEUA/Unicamp, protocol #4272-1, 2016) at the Campinas State University (Unicamp)—Campinas, SP, Brazil. The animals were kept in a suitable environment under 23 ± 2°C, 50 ± 10% relative humidity, and a light-dark cycle from 7:00 a.m. to 7:00 p.m. At 11 weeks of age, the animals were mated, and the day that sperm were seen in the vaginal smear was designated day one of pregnancy. Then, dams were maintained *ad libitum* throughout the entire pregnancy on an isocaloric rodent laboratory chow with either standard protein content [NP, n = 10] (17% protein) or low protein content [LP, n = 10] (6% protein) diet (Langley-EvansGardner and Welham, 1998). In the current study, we established the age of 62 weeks to perform examinations. Morphological and molecular analyses were performed in NP and LP male offspring (rats from different mothers). The NP and LP maternal food consumption were determined daily (subsequently normalized for body weight), and the bodyweight of dams was recorded weekly in both groups. Male pups from the NP (n = 49) and LP (n = 51) different litters were weighed at birth. After weaning, the offspring of both experimental groups were followed and maintained under normoprotein rodent laboratory chow weekly until the 62nd week of life. Bodyweight (BW) was obtained on the 48th, and at the 62nd week, rats were euthanized for experimental procedures. Langley-Evans & Sculley (2006), whose experimental protocol was very similar to the present one, have shown that male rodents submitted to gestational protein restriction (9%) had a mean survival (identical to the median) of 69 weeks. Based on this study, we established the ages of 48 and 62 weeks to be evaluated experimentally. In addition, the heart left (LV) and right (RV) ventricles and adipose tissue were weighed. Left ventricle samples from 62-week-old animals were collected for mRNA sequence, immunohistochemistry, and western blot.

Blood pressure measurement

Briefly, the indirect tail-cuff blood pressure measurement method was determined by an electro-sphygmomanometer combined with a pneumatic pulse transducer/amplifier. The Windaq software graphically established the blood pressure from the Automatic Cuff Inflation Pump, ITC Life Science Inc.®. The systolic arterial pressure was measured in a random sample of conscious animals, gently restrained in specific containers purchased by the device manufacturer at 32, 40, 48, 52, and 62-week-old NP (n = 15) and compared to age-matched LP (n = 25) male offspring. Measurements were conducted during the same period of the day. The mean of three consecutive readings was taken as the blood pressure. This indirect approach allowed repeated measures with close correlation (correlation coefficient = 0.975) compared with direct intra-arterial recording.

Collagen content, myocyte area, and left ventricle thickness

Male 62-week-old LP (n = 5) and age-matched NP (n = 5) offspring from different litters were anesthetized with a mixture of ketamine (50 mg/kg body weight, i.p.) and xylazine (1 mg/kg body weight, i.p.). The LV was perfused with a heparinized saline solution (1%) followed by a 4% (w/v) paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). After perfusion, the LV was dissected, fixed for 24 h in the paraformaldehyde solution, and then embedded in the paraplast (Sigma-Aldrich, United States). Five-micrometer-thick sections were stained with hematoxylin and eosin (HE) or picrosirius red. The measurements were performed from digital images collected by a video camera attached to an Olympus microscope (× 40 magnification lens), and the images were analyzed by ImageJ software. The cross-sectional area was measured with a digital pad. Selected cells were transversely cut so that the nucleus was in the center of the myocyte and determined an average of at least 30 myocytes per animal. The left ventricular interstitial collagen volume fraction was marked by picrosirus-red and stained with a fast green was calculated; as the ratio between the connective tissue area and connective tissue plus myocyte areas from 30 microscope fields of digitalized images of each animal. Perivascular collagen was excluded from the morphological study analysis. The stained LV slides images were also scanned and analyzed using the ImageJ software to measure wall thickness and lumen area. Also, the cross-sectional myocyte slices from 62-week-old NP (n = 5) and LP (n = 5) offspring from different litters were stained with hematoxylin and eosin. The selected cells, measured with a digital pad, were transversely cut so that the nucleus was in the center of the myocyte and determined an average of at least 30 myocytes per animal. The CellSens Dimension program estimated the concentration of collagen present in the groups.

Adiposity index

Forty-eight-week-old and 62-week-old male NP (n = 15) and age-matched LP (n = 15) progeny from different litters were anesthetized by isoflurane. The adipose tissue was then dissected and weighed. Total body fat was measured as the sum of the following individual fat pad weights: epididymal fat + retroperitoneal fat + visceral fat. The adiposity index was calculated as (total body fat/final BW) x100.

Total RNA extraction and RNA sequencing

Based on the protocol developed and revised by Chomczynski & Sacchi (2006), LV total RNA from 62-week-old male LP (n = 5) offspring compared to age-matched NP progeny (n = 5) from different litters was extracted using the

TABLE 1 Primer sequences forward and reverses (5′-3′).

	Primer Fwd (5'-3')	Primer Rev (5'-3')
ADRB 1	CGCTGCCCTTTCGCTACCAG	CCGCCACCAGTGCRGAGGAT
CBP	TGGAGAAGCAAGGAGGTC	GCGGCGTAAGGAAGAGAAC
IRS2	GTATTAGATAAGGAACCAAGAGGC	AAAGTAACAGGAGAAATGACAGCA
RAP 1	TGCTTGAAATCCTGGATACTG	AGCCTTGTCCGTTCTTCATGTAC
P300	GACCCTCAGCTTTTAGGAATCC	TGCCGTAGCAACACAGTGTCT
TXN2	CGGACATTTCACACCACCAGAG	CCGTGCTGTTTGGCTACCATC
GARDH	CCTTCATTGACCTCAACTACATG	CTTCTCCATGGTGGAAGAC

Trizol extraction method. A High Capacity cDNA reverse transcription kit (Life Technologies, United States) was used for the cDNA synthesis. Two µl cDNA (40 ng/µl) was added to containing specific primers (Table 1) and the SYBR® Green JumpStartTM Taq ReadyMixTM (catalog number S4438). The total RNA quantity, purity, and integrity were assessed previously for miRNAs expression analysis (Assalin et al., 2019; Sene et al., 2013, 2018, 2021). Before sending the samples for sequencing, a library was built for each sample, following the protocol provided by the manufacturer of the TruSeq Stranded mRNA kit (Illumina) (https://www.illumina.com/products/by-type/sequencing-kits/library-prepkits/truseq-stranded-mrna.html). Sequencing was performed on the Hiseq platform at LaCTAD—Central Laboratory for High-Performance Technologies, UNICAMP, Campinas, Brazil. Reads were aligned to the *Rattus norvegicus* Ensembl Rnor 5.0 assemble genome using the STAR aligner tool.

(https://github.com/alexdobin/STAR, V 2.7.0). Data analysis was performed using the program for normalization and statistical analysis DESeq2 (DESeq2 package version: 1.35.0). (Data available in NCBI's Gene Expression Omnibus, accessible through GEO Series accession number GSE188836 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188836). Data are expressed as the mean ± standard deviation, and the estimates of dispersion and logarithmic fold changes incorporate data-driven prior distributions in NCBI's Gene Expression Omnibus. A list of all differentially expressed genes in the treated group compared with the control group was generated. The transcriptome was analyzed using DAVID Bioinformatics Resources 6.8—Laboratory of Human Retrovirology and Immunoinformatics (LHRI) (https://david.ncifcrf.gov/). Genes with significantly altered expression, assuming $p < 0.05$, were shown in the current study and analyzed for their function and possible action pathways. Functional analysis was performed based on pertinent literature on aging and cardiac disease. Gene function was investigated based on published articles from peer-review indexed journals available in scientific databases by search engines such as PubMed (www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (https://apps.webofknowledge.com) (Supplementary Table S1).

TABLE 2 List of antibodies used manufacturer and catalog number.

Antibody	Manufacturer	Catalog
AT1	Santa Cruz	Sc-31181
AT2	Santa Cruz	sc-9040
AKT	Santa Cruz	sc-8312
pAKT	Santa Cruz	sc-101629
CREB	Cell Signaling	9104
pCREB	Cell Signaling	9191
ERK1/2	Cell Signaling	4370S
Na/KATPase	Santa Cruz	sc-21713
NOS1	Santa Cruz	sc-648
SOD2	Santa Cruz	sc-30080
STAT3	Santa Cruz	sc-483
pSTAT3	Santa Cruz	sc-8001
AMPK	Cell Signaling	25321
pAMPK	Cell Signaling	25351
JAK2	Santa Cruz	sc-278
pJAK2	Cell Signaling	3775

The RNA-Seq validation was performed using cDNA analysis for real-time PCR assays (RT-qPCR). The PCR cycles were performed in the StepOne Plus equipment (Life Technologies, United States) under the following conditions: 94°C for 2 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. Ct values were converted to relative expression values using the ΔΔCt method. The offspring heart data were normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) used as endogenous control and reference gene.

Immunoblotting—LV of 62-week-old male NP ($n = 5$) and LP ($n = 5$) offspring from different litters were used to perform the protein level analysis by western blot. LV was homogenized in solubilization buffer (100 mM Tris-hydroxymethyl-aminomethane pH7.4, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM ethylenediaminetetraacetic acid, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml aprotinin) using a polytron PTA 20 S

generator (model PT 10/35) Brinkmann Instruments, Westbury, N.Y. United States) at maximum speed. The tissue extracted was incubated with 10% volume Triton-X 100 and then centrifuged at × 22.050 g at 4°C for 40 min. Supernatant proteins were quantified using the Biuret method. The samples were mixed with Laemmli buffer containing 100 mM dithiothreitol, heating at 95°C for 5 min. Each sample (120 ug of protein) was subjected to gel electrophoresis in Bio-Rad mini gel apparatus (Mini-Protean SDS-Page, Bio-Rad Laboratories, Hercules, C.A. United States). The proteins separated in the gel were transferred to the nitrocellulose membrane and performed for 90 min at 120 V using the BioRad® mini gel electro-transfer equipment. After the transfer, the membrane was stained with Ponceau for normalization and later prepared to be immersed in the primary antibody of interest (Table 2) and, subsequently, immersed in the secondary antibody solution. Non-specific protein binding was reduced by incubating the membrane for 1 h at ambient temperature in blocking buffer (5% bovine serum albumin (BSA), 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). A Supersignal West Pico Chemiluminescent Substrate chemiluminescence kit (Thermo Scientific®) was used to reveal the membranes. The bands were quantified by optical densitometry using the software Un-Scan-It gel 6.1.

Data analysis

Data was previously tested to assess the normality of distribution frequency and equality of variance by the Shapiro-Wilk and the Levene test. Data are expressed as the mean ± standard deviation. Comparisons between two groups were performed using Student's t-test when data were normally distributed and the Mann-Whitney test when distributions were non-normal. Comparisons between two groups through the weeks were performed using 2-way ANOVA for repeated measurements test, in which the first factor was the protein content in the pregnant dam's diet and the second factor was time. The mean values were compared using Tukey´s post hoc analysis when the interaction was significant. Significant differences in the transcriptome were detected using a moderated *t*-test. Data analysis was performed with Sigma Plot v12.0 (SPSS Inc. Chicago, IL, United States). The significance level was 5%.

Results

The dam's body masses were not different in the LP compared to NP groups during the whole three weeks of gestation (1st wk: LP: 276 ± 25 vs. NP 267 ± 25; 2nd wk: LP: 303 ± 26 vs. NP 290 ± 29; 3rd wk: LP: 325 ± 37 vs. NP 325 ± 29). The birth weight of male LP progeny (n = 51, from 10 different mothers) was significantly smaller than that observed in male NP (n = 49, from 10 other mothers) offspring (LP: 6 ± 0.6 vs. NP: 6.4 ± 0.7, p = 0.0028). After 7 days from delivery, the LP and NP offspring body masses are similar (LP: 15.3 ± 2 vs. NP: 15.8 ± 1.6, p = 0.1, n = 35). As shown in Figure 1, the systolic arterial pressure was significantly higher in LP than NP offspring beyond 48 weeks (p = 0.001) until the tend experimental time. At 48 weeks of age, the body mass of LP (n = 51) and NP (n = 49) progenies were also equal (Table 3). However, at 62 weeks of age, the LP offspring showed lower body mass than the NP rats (Table 3). The heart, RV, and LV masses from LP (n = 15) were higher than in NP (n = 15) offspring at 62 weeks of age (Table 3). The LP offspring presented a significantly enhanced adiposity index (n = 15) when compared to those found in the NP group (n = 15) at 48 weeks of age (Table 3). At 62 weeks of age, the adiposity index from LP (n = 15) was unchanged compared to NP (n = 15) progeny (p = 0.7) (Table 3). The LV lumen plotted area was not altered in LP; however, the heart wall thickness was significantly enhanced at 62-week-old LP (Table 3) compared to age-matched NP offspring. The plotted area of cardiac myocyte and the immunostained collagen content increases significantly in the 62-week-old LP (LP: 9.364 ± 4.7 in % of collagen per LV area, n = 97) progeny compared to the age-matched NP (NP: 7.830 ± 3.982 in % of collagen per LV area, n = 96, p = 0.0303) (Figure 2).

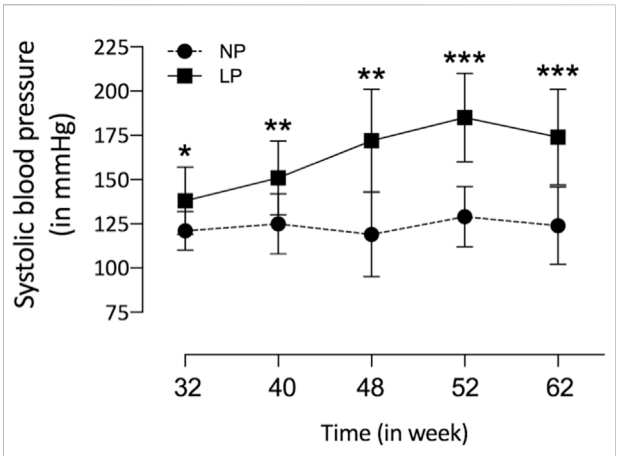

FIGURE 1
Tail systolic arterial pressure from NP (n = 15) compared to age-matched LP (n = 25) male offspring from 32nd to 62nd weeks of age was measured by an indirect tail-cuff method using an electrosphygmomanometer. The results were expressed as mean ± SD, and the two-way ANOVA statistical analysis was performed. *p < 0.05 **p < 0.01, ***p < 0.001.

The literature findings categorize genes implicated in cardiac function using differentially expressed genes identified by transcriptome analysis (Supplementary Tables S1,S2). These specified genes expression correlated with cardiac function, hypertrophy, fibrosis, inflammation, oxidative stress, and adrenergic receptors (Supplementary Tables S1,S2; Figure 3). However, when the current study analyzed the LV global

TABLE 3 The table depicts the progeny's bodyweight, heart and left (LV), right ventricles (RV) masses, adiposity index, LV lumen, and wall measures.

	48 weeks		62 weeks	
	NP	LP	NP	LP
Animal mass (g)	567	583	633±37	580±42***
Heart/animal mass x 100 (g)	0.19±0.001	0.19±0.03	0.18±0.01	0.2±0.01**
RV/animal mass x 100 (g)	0.039±0.003	0.038±0.01	0.03±0.006	0.035±0.006*
LV/animal mass x 100 (g)	0.15±0.0001	0.15±0.02	0.15±0.01	0.16±0.01*
Adiposity index x 100	5.6±1.5	5.9±2.5*	6.5±1.5	6.4±1.6
LV lumen (pixel3)	—	—	0.03±0.008	0.03±0.005
LV wall thickness (pixel3)	—	—	0.065±0.008	0.075±0.007*

Bodyweight (BW) was obtained on the 48th and 62nd week progeny (NP; n=49) and (LP; n=51) from different litters,For heart, RV, LV masses, and adiposity (n=15 for each experimental group). LV lumen and wall thickness (n=5 for each experimental group). Comparisons betweeen experimental were performed using 2-way ANOVA for repeated measurements or Student's test, *p< 0.05, ***p< 0.01, ***p< 0.001

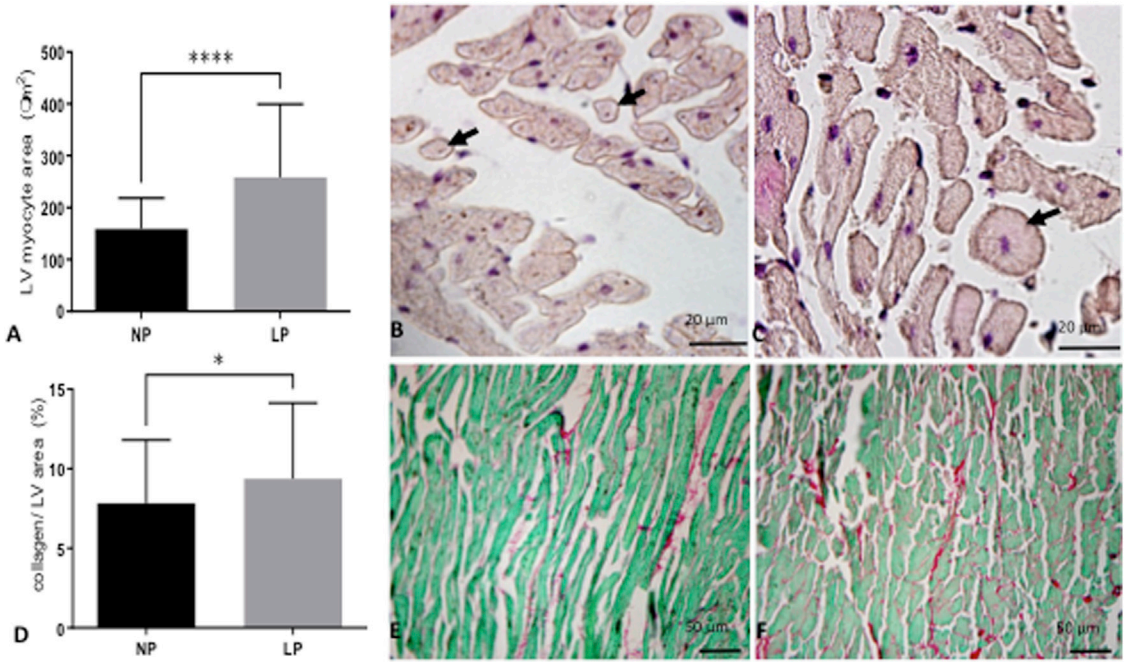

FIGURE 2
In A, a graphic of LV cardiomyocytes (arrow) area (NP: 174 fields; LP: 199 fields from n = 5) and representative micrographs of NP offspring **(B)** and age-matched LP progeny **(C)** LV tissue stained with hematoxylin-eosin (HE). In D, depicted LV collagen content (100 fields for each group from n = 5) and picrosirius fast green stained LV tissue from NP **(E)** and LP **(F)** offspring. The results were expressed as mean ± SD, and Student's t-test performed the statistical analysis. *p < 0.05, ****p < 0.0001.

transcriptome, the LP (n = 5) progeny presented 137 differentially expressed genes (p < 0.05) compared to NP (n = 5) offspring being that 13 of these had unknown transcripts Supplementary Tables S1,S2). Supplementary Figures S1,S2 depicted the PCA plot to illustrate the samples' relatedness and a volcano plot representing the magnitude of changes in RNA-Seq.

Remarkably, the altered thioredoxin 2 (TXN2) RNA sequencing was 22% downregulated in the LP group compared to NP. Furthermore, as shown in Figure 3, significant changes in 8 other mRNAs related to oxidative stress pathways were identified. Also, they are related to insulin resistance, metabolism of carbohydrates, lipid, and

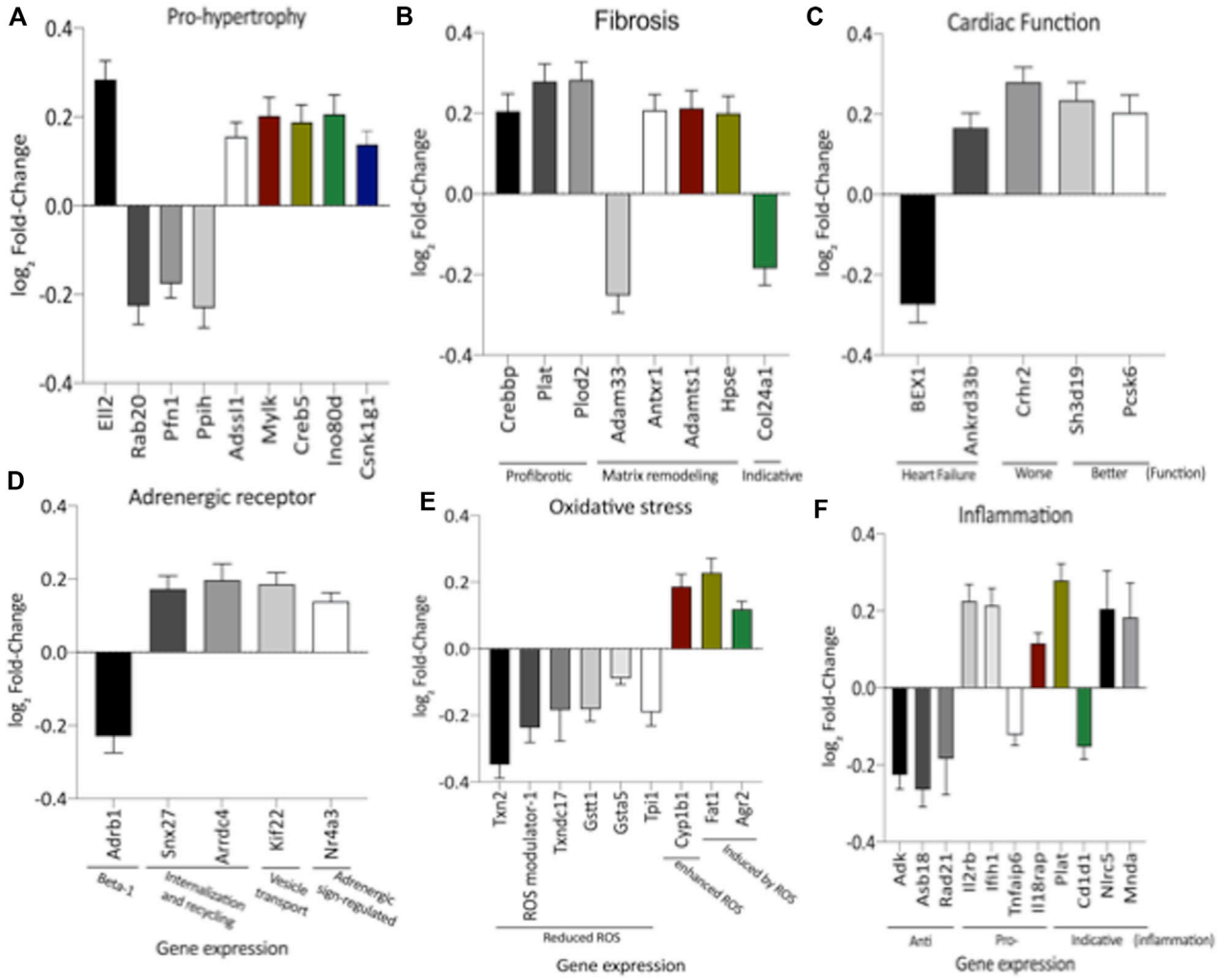

FIGURE 3
Expression of genes significantly altered, grouped by their hole in hypertrophy **(A)**, interstitial fibrosis **(B)**, cardiac function **(C)**, adrenergic receptor signaling **(D)**, oxidative stress **(E)** and inflammatory activity **(F)**. This analysis was performed based on relevant literature on aging and cardiac disease (the gene's abbreviations and references are found in Supplementary Table S1). The results were expressed as mean ± SEM.

enzymes (Supplementary Table S1; Figure 4), apoptosis, DNA structure, autophagy, calcium metabolism, oncogenes (Supplementary Tables S1,S2; Figure 5), protein synthesis, cell membrane compound, ion channels, and cytoskeleton (Supplementary Tables S1,S2; Figure 5). The present study validated that most mRNAs of the cAMP signaling pathway and ADRB1, CREBBP, and RAP1 were statistically different when comparing LP progeny and age-matched NP offspring (Figure 6) with no change in IRS2 and p300 expression. The investigated blotting nitric oxide synthases (NOS1), and superoxide dismutase-2 (SOD2) LV myocyte levels did not demonstrate any change in NP and LP offspring for these proteins (Figure 7); however, although not statistically significant (p = 0.55), NOS1 content was higher in LP animals. Regarding the cAMP and oxidative stress pathways cross-talking, despite the increase in mRNAs encoding CREB, the encoding β-adrenergic receptor is significantly decreased. However, the investigated blot levels of phosphorylated CREB were elevated considerably in LP offspring than in NP progeny (Figure 8). Concerning other proteins that mediate CREB expression, the study found unchanged ERK-1 and 2. However, a significant reduction in phosphorylated AKT was observed in LP progeny (Figure 8).

The present study also studied the expression of the Ang-II receptors. Despite not observing significant changes in AT1, the expression of the AT2 receptor was considerably reduced in LP progeny (Figure 7), resulting in a substantial enhancement in AT1/AT2 ratio. Also, the current finding observed a significant

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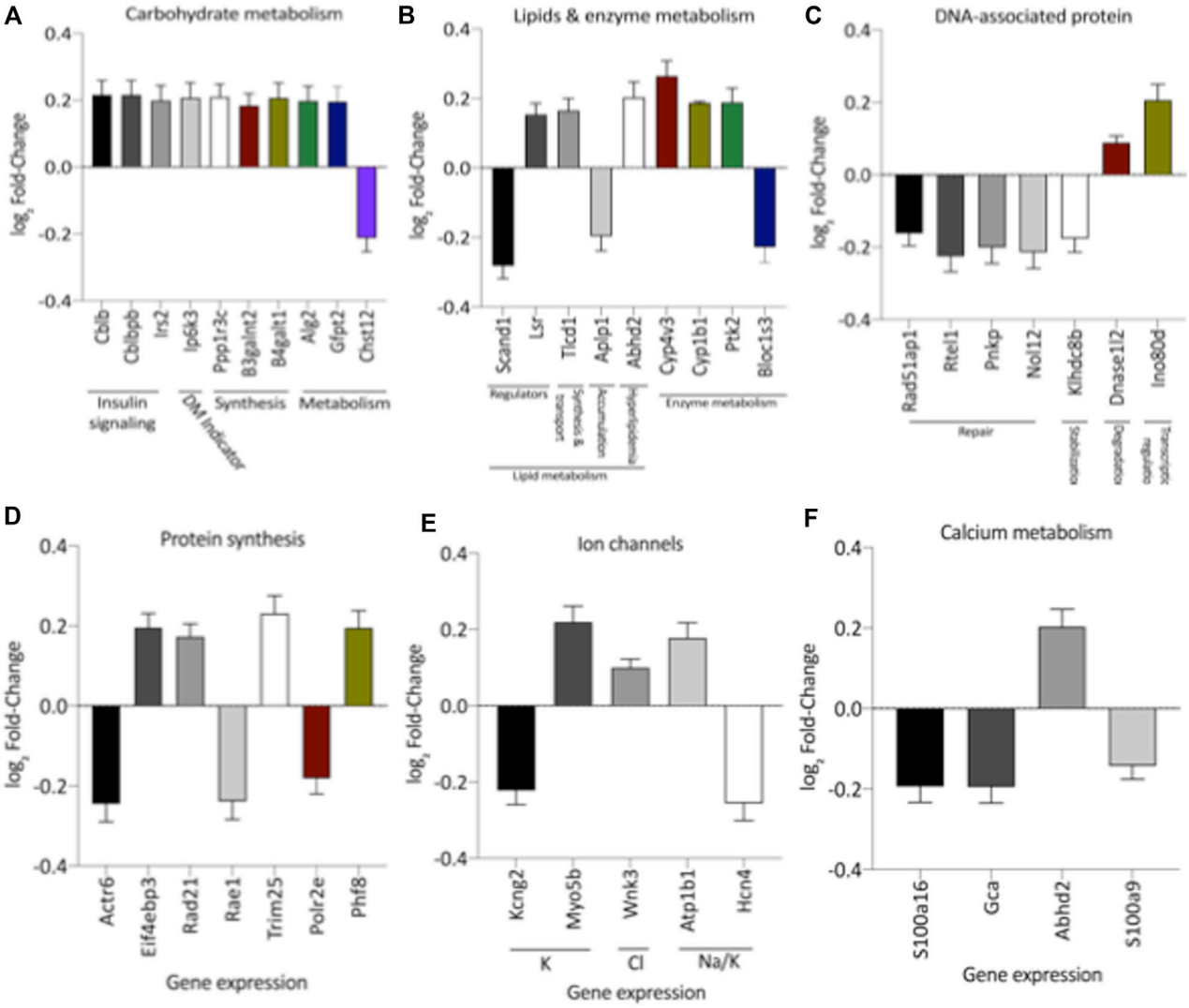

FIGURE 4
Expression of genes significantly, grouped by their role altered in carbohydrate **(A)**, lipids metabolism **(B)**, DNA-associated protein **(C)**, protein synthesis **(D)**, and Ion **(E)** and Calcium Channels **(F)**. These analyzes were performed based on pertinent literature on aging and cardiac disease (the gene's abbreviations and references are found in Supplementary Table S1). The results were expressed as mean ± SEM.

reduction of the mRNA encoding PKA, which positively regulates the expression of Na/K-ATPase in LP compared to the NP group. However, the increased Na-K ATPase transcript expression in gestational protein-restricted offspring was not confirmed by an immunostaining study of the content of this sodium pump in heart tissue of animals with 62-week of life compared to age-matched controls (Figure 8). Figure 9 depicted a schematic representation of the cyclic AMP and associated pathways investigated and biological response disorders in LV from 62nd weeks of age male rats from maternal restricted-protein intake. The cAMP-signaling path (Figure 9) was recognized by transcriptome analysis using DAVID Bioinformatics Resources 6.8—Laboratory of Human Retrovirology and Immunoinformatics (LHRI) (https://david.ncifcrf.gov/). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE188836 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188836).

Discussion

The current study confirms previous data showing that gestational protein restriction is associated with reduced embryo/fetal growth and low birth weight compared to NP

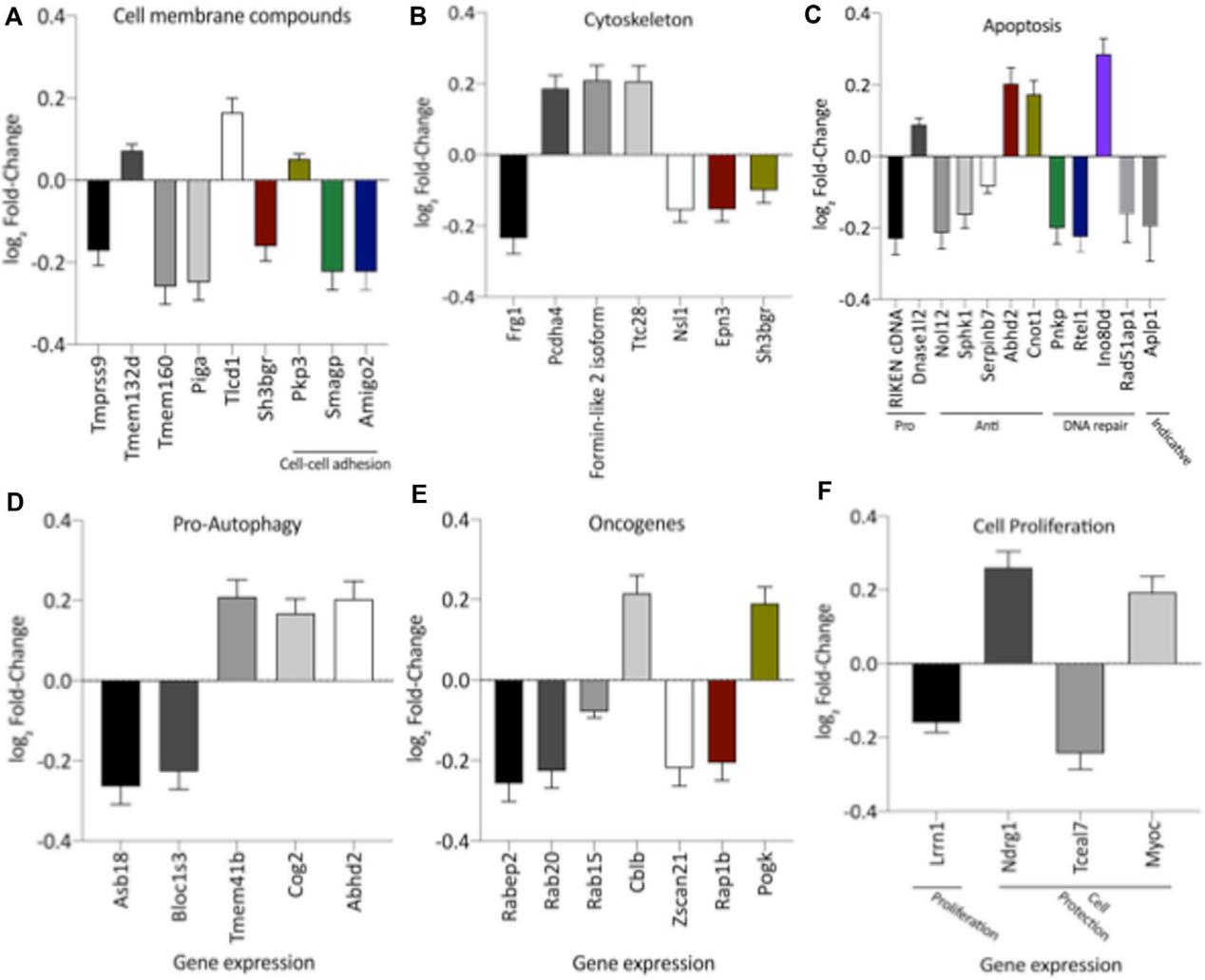

FIGURE 5
Expression of genes significantly altered, grouped by their participation in cell membrane compounds **(A)**, cytoskeleton **(B)**, apoptosis **(C)**, autophagy **(D)**, oncogenes **(E)**, and cell proliferation **(F)**. The analysis was performed based on pertinent literature on aging and cardiac disease (the gene's abbreviations and references are found in Supplementary Table S1). The results were expressed as mean ± SEM.

offspring (Edwards et al., 2001; Fernandez-Twinn, 2004; Corstius et al., 2005; Mesquita et al., 2010a, Mesquita et al., 2010b; De Lima et al., 2013; Lamana et al., 2021). However, the LP progeny mass body recovered from one week of life, with no significant difference related to age-matched NP progeny. The rapid body mass gain is known as catch-up growth (Cianfarani et al., 1999; Zohdi et al., 2015), a well-established additional risk factor for several diseases in adult life (Ong et al., 2000; Forsén et al., 2000; Law et al., 2002; Tarry-Adkins et al., 2009). This effect leads to gender-related disorders in blood pressure, glucose metabolism, and anxiety-like behaviors in male adult progeny compared to female offspring (Kwong et al., 2000; Gillette et al., 2017). Also, it is essential to state here that sex hormones determine sexual phenotype dimorphism in the fetal-programmed disease model in adulthood by changes in the long-term control of neural, cardiac, and endocrine functions. Specific hormones and the estrus cycle interfere with behavioral, hemodynamic, and systemic water and ion homeostasis in female rodents. Thus, the present study was limited and performed on male rats considering the findings above to eliminate interferences due to gender differences (Kwong et al., 2000; Gillette et al., 2017). However, additional research with long-term follow-up and cross-fostering, including behavioral tests in female offspring, would help specify the nature of some protein-restriction effects. Previous studies have demonstrated that the multifactorial onset of the blood pressure increase in LP offspring begins at the 8th week of life, remaining elevated beyond the 16th week of age (Edwards et al., 2001; Mesquita et al., 2010a; Mesquita et al.,

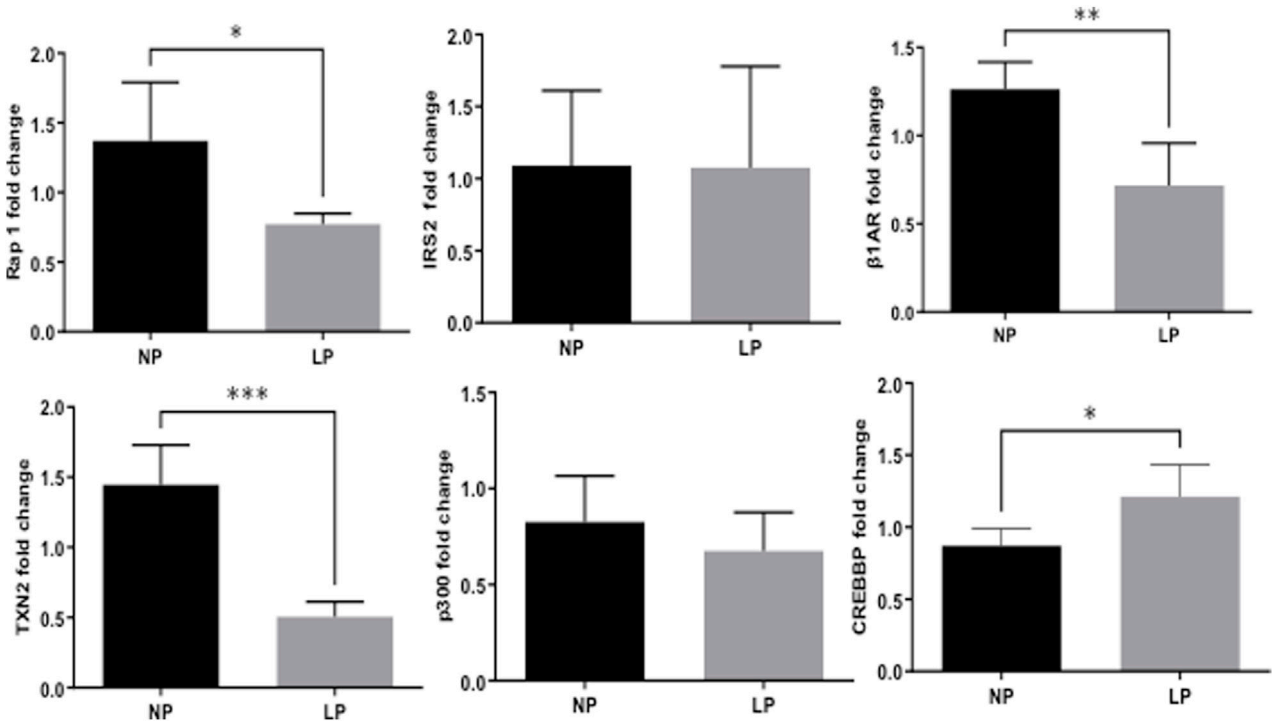

FIGURE 6
Fold-change of mRNA expression in LP offspring (n = 5) compared to age-matched NP progeny (n = 5).

2010b; Mariano et al., 2021). As confirmed in the present study, data from the 32nd to the 62nd week of life showed that the arterial pressure in the LP progeny remained consistently higher in LP offspring than in NP progeny. The increased blood pressure in LP offspring may respond to several factors such as the reduced nephron number, increased tubular Na-K ATPase expression, and sympathetic renal nerve activity, phenomena associated consequently with decreasing urinary sodium excretion and elevated blood pressure (Mesquita et al., 2010a,b; Lamana et al., 2021; Custódio et al., 2017; Mariano et al., 2021). The reduced nephron number promotes blood overflow and glomerular hyperfiltration in the remaining nephrons. These effects, in turn, naturally promote glomerular fibrosis and senescence, keeping the progressive cycle of blood pressure enhancement (Langley-Evans et al., 1999; Vehaskari et al., 2001; Lucas et al., 2001).

Data available in the literature are controversial regarding cardiac mass data. In the current study, 62-week-old LP offspring confirmed a significant reduction in body mass, in parallel with a reciprocal increase in the heart and isolated ventricles weight when normalized by animals' body mass. The discrepancy in results from prior studies may be related to using different animal strains, protein restriction levels, and the duration and growth time when the protein restriction was implemented (Zohdi et al., 2015). So, studies with Wistar Kyoto lineage subjected to protein restriction during the whole pregnancy period cause a significant reduction in heart mass (Corstius et al., 2005). Still, the dams of Wistar Kyoto rats, maintaining restricted protein intake during breastfeeding, showed uneven results, characterized by unchanged or increased heart mass compared to control offspring (Lim et al., 2010; Jackson et al., 2002). A restricted protein intake (6% protein) prior study in our laboratory demonstrated an increased left ventricular mass and heart volume in 16-week-old LP offspring (Silva et al., 2013; Assalin et al., 2019). However, the present study did not confirm the enhanced cardiac mass in 62-week-old LP progeny.

Studies have demonstrated that enhanced collagen content, in turn, reduces myocardial elasticity and compliance, causing decreased contractility and, consequently, myocardial dysfunction (Capasso et al., 1990; Burlew & Weber, 2002; Assalin et al., 2019). It has been observed that collagen is the main structural protein in the extracellular matrix, providing support and rigidity to the myocardium (Xu et al., 2006). Its deposition increases with aging (Weber, 1989; Eghbali et al., 1988). In 16-week old gestational protein-restricted progeny, we showed a striking increase in the cardiomyocyte cross-sectional area associated with enhanced interstitial collagen deposition in the LV (Assalin et al., 2019). In the present study, evaluating the

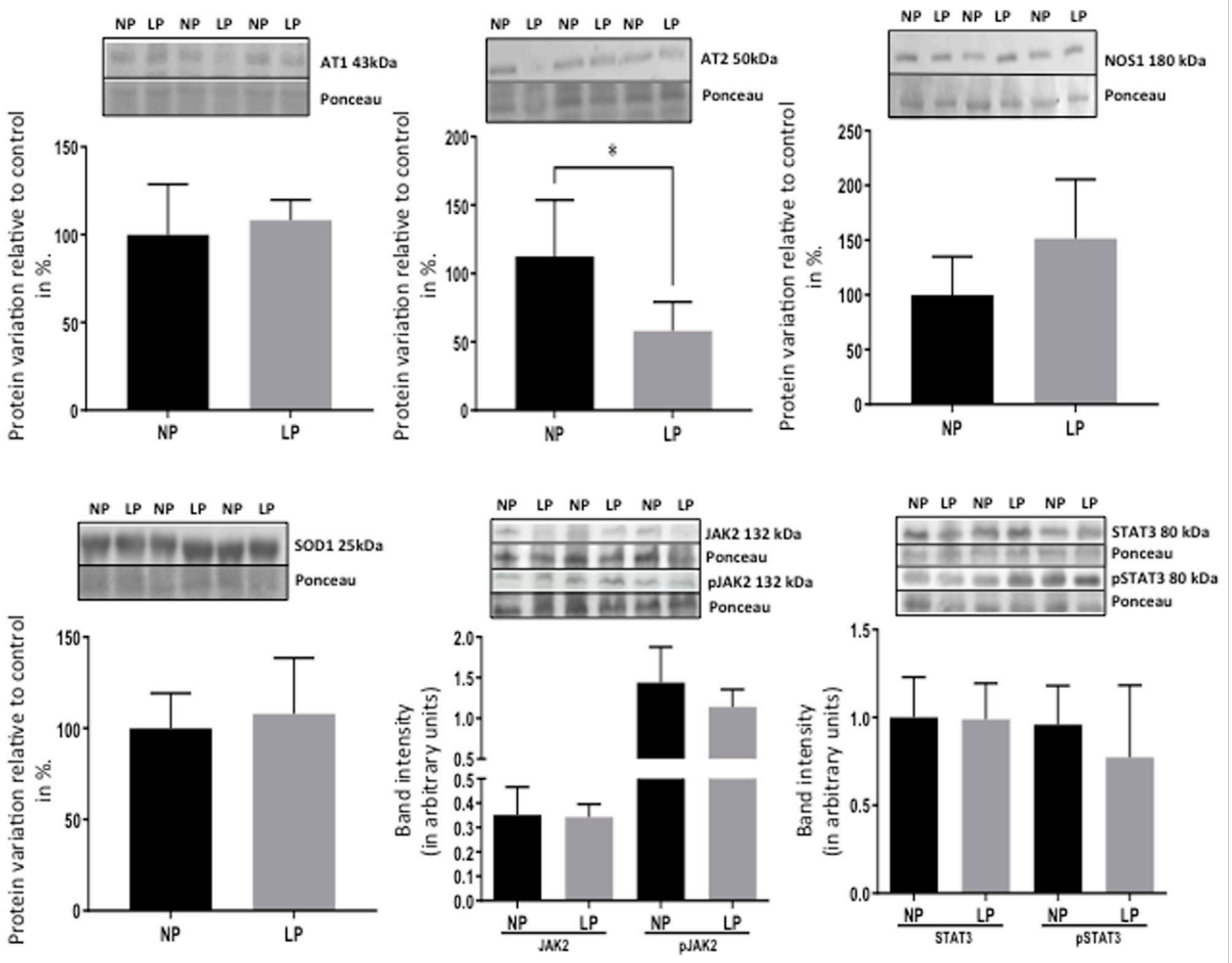

FIGURE 7
Effect of maternal protein restriction on AT1, AT2, NOS1, SOD2, JAK2, pJAK2, STAT3, and pSTAT3 proteins, measured using immunoblotting analysis in the isolated LV. The protein variation results from LP progeny were expressed as mean ± SD and relative to control (NP offspring), assigning a value in %). The statistical analysis was performed by Student's t-test when comparing only two samples of independent observations; only one offspring of each litter was used for immunoblotting experiments. *$p < 0.05$.

ventricle collagen content in 62-week-old LP progeny, a significant enhancement deposition was also found (Lim et al., 2006; Xu et al., 2006; Menendez-Castro et al., 2011; Menendez-Castro et al., 2014; Assalin et al., 2019). Additionally, we demonstrated significant hypertrophy of cardiomyocytes in 62-week-old LP offspring, as previously observed by other authors (Morrison et al., 2007; Bubb et al., 2007; Louey et al., 2007). This finding can be taken as an immediate adaptive myocardial remodeling process in response to pressure overload, humoral factors, or compensatory mechanisms resulting in a contraction deficit (Sohal et al., 1993; Tarry-Adkins et al., 2010; He et al., 2015). Notably, previous studies confirming our data have demonstrated that gestational protein restriction reduces the number of cardiomyocytes (Corstius et al., 2005; Xu et al., 2006).

We may also assume that the hypertrophied cardiomyocytes may result from arterial hypertension or compensatory mechanisms for temporarily maintaining adequate cardiac output in 62-week-old LP offspring. In the current study, the reduced myocyte number may attenuate striking an expected enhanced heart mass in the LP offspring despite the cardiomyocyte hypertrophy.

CREB is a transcription factor responsible for activating genes in response to external factors that lead to cardiac hypertrophy and fibrosis (Kumar & Pandey, 2009). Regarding the cAMP signaling pathway, a significant increase in the expression of mRNAs encoding CREB5 and CREB binding protein (CREBBP) was observed in 62-week-old LP progeny (Figure 3). However, two of the mRNAs encoding CREB pathway meditators showed a significant decrease (PKA and Rap1) in LP

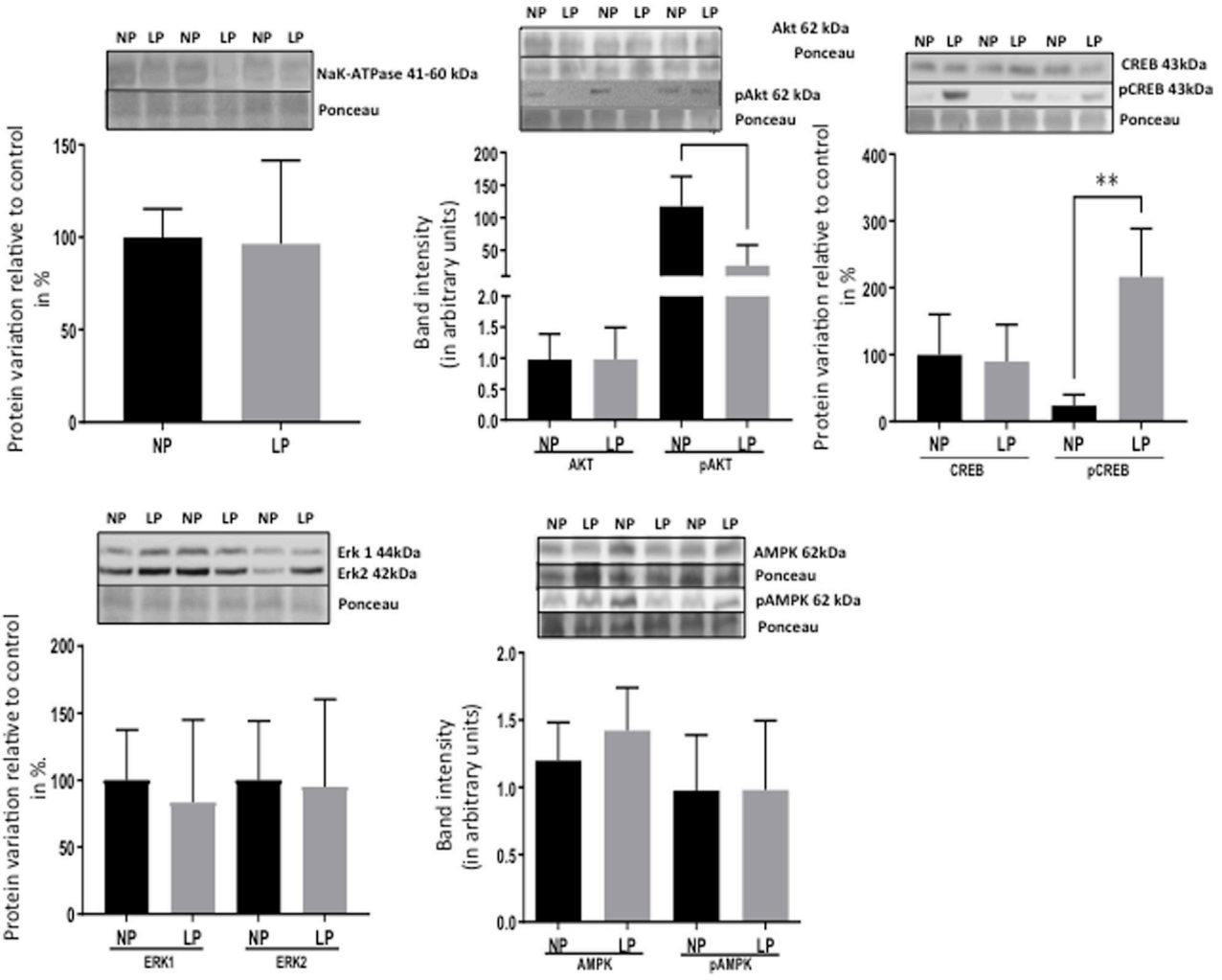

FIGURE 8
Effect of maternal protein restriction on ERK 1/2, Na-K ATPase, AKT, pAKT, CREB, pCREB, AMPk, and pAMPk proteins, measured using immunoblotting analysis in the isolated LV. The statistical analysis was performed by Student's t-test when comparing only two samples of independent observations; only one offspring of each litter was used for immunoblotting experiments. The protein variation results from LP progeny were expressed as mean ± SD and relative to control (NP offspring), assigning a value in %. *$p < 0.05$; **$p < 0.01$.

offspring compared to the NP group. By blot analysis, the current study showed a substantial increase in phosphorylated CREB in LP animals, suggesting that additional pathways besides the cAMP could activate CREB expression.

The current study analyzing type 1 and 2 Ang-II receptors establishes that an increased AT1/AT2 ratio causes receptor imbalance, prevailing an enhanced type 1 Ang-II receptor stimuli, leading to increased CREB phosphorylation. Also, changes in renin-angiotensin-system (RAS) components characterized by raised renal angiotensin AT1/AT2 receptors ratio expression could explain the high blood pressure compared to NP rats (Mesquita et al., 2010a,b). By the way, prior studies have demonstrated that activation of type-1 Ang-II receptors leads to CREB phosphorylation throughout a signaling pathway involving PI3K/AKT and Erk1/2, which results in cardiac hypertrophy and associated fibrosis (Cammarota et al., 2002; Sahar et al., 2007; Shanmugam et al., 2011). However, considering mediators involved in CREB phosphorylation, the present data did not show significant ERK1/2 protein blot detection change. Thus, we may suppose that increased phosphorylated CREB could be associated with non-adaptive hypertrophy, fibrosis, and reduction in the heart myocytes, presumably by activating Type 1 Ang-II receptors phosphorylation but not by ERK1/2 and other MAPKs cascades protein expression.

Phosphorylated Akt is an essential intermediate protein in the insulin pathway activation. Otherwise, a substantial reduction in phosphorylated Akt was observed in the left

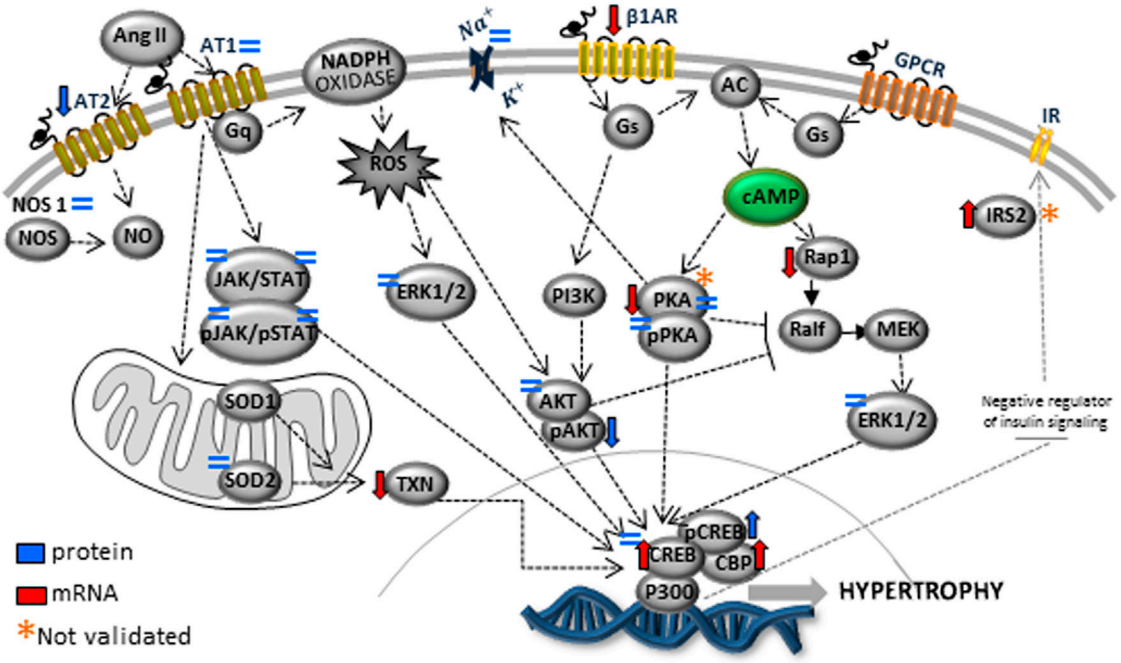

FIGURE 9
The picture depicted a schematic representation of the cyclic AMP and associated pathways investigated and biological response disorders in LV from 62nd weeks of age male rats from maternal restricted-protein intake.

ventricle of LP progeny compared to age-matched NP. We may suppose that the considerable increase in mRNA encoding Cblb (Casitas B-lineage lymphoma b), an ubiquitin E3 ligase that specifically degrades IRS1, may also be involved in pAkt reduction (Nakao et al., 2009). Also, inositol hexakisphosphate kinase-3 (IP6K3) generates inositol pyrophosphates that regulate several cellular functions. Its expression is increased in the skeletal muscle of diabetic mice. It was also observed that the IP6K3 deletion extended mice's life span, leading authors to suggest a new role for this protein in metabolic control and survival lifetime (Moritoh et al., 2014). Here, it was demonstrated that in LV of the LP progeny, the mRNA encoding IP6K3 is significantly increased related to reduced span time of life in LP progeny compared to NP offspring, as observed in a previous study in our Lab (Grigoletti-Lima et al., 2022). In this way, cardiac aging is characterized by an increased inflammatory process and oxidative stress.

The stimulated type 1 Ang-II receptors in gestational protein-restricted offspring could further promote vasoconstriction, oxidative stress, and inflammation (Cheatham et al., 1994; Brownsey et al., 1997; Araújo et al., 2005). The assertion above is supported by changes in the expression of mRNAs encoding proteins related to increased oxidative stress and inflammation in the LP offspring compared to the NP progeny. Previous studies corroborate these findings showing that gestational protein restriction leads to greater susceptibility to oxidative stress (Langley-Evans & Sculley, 2005; Tarry-Adkins et al., 2010; He et al., 2015) and inflammatory process (Tuchscherer et al., 2012; Senna et al., 2015). In the current study, when analyzing the gene sequencing, we can observe that the most altered mRNA is the coding for Txn2, which is decreased in LP progeny. TXN2 is responsible for reducing reactive oxygen species (ROS), but we did not observe any significant difference in SOD2 expression. Although not substantial, this study also presented an increased expression of NOS1 in the LV of LP offspring. Several biological pro-inflammatory cytokines effects lead to heart failure in human and experimental models (Maier et al., 2012; Butts et al., 2015; Fenton & Parker, 2016; Deng et al., 2016). Here, in LP progeny, the LV showed the expression of four mRNAs encoding to beta-2 interleukin receptor subunit, domain 1 of interferon-induced helicase C, TNF alpha protein 6 induced, and interleukin receptor 18 accessory proteins. This data supports an inflammatory activity process in the LV of 62-week-old LP progeny. Thus, we may hypothesize that molecular effects underlying the innate immune response might be implicated in inflammation and heart disease.

The current study also demonstrated a significant reduction of mRNA encoding BEX1 (brain-expressed X-linked protein 1) in the LV of LP progeny. The expression of the BEX1 gene is linked to heart failure and is associated with gene expression related to heart disease (Accornero et al., 2017). So, the study also

found an increased mRNA to encode CARP (cardiac ankyrin repeat protein) in the heart of LP offspring. CARP is predominantly expressed in cardiac muscle and is related to cardiomyocyte hypertrophy before the development of heart failure. A significant increase in CARP mRNA and protein expression in left ventricular tissue patients with end-stage heart failure is observed (Zou et al., 1997; Zolk et al., 2002).

In the heart, G protein-coupled receptors (GPCRs) respond to extracellular stimuli and are involved in fibrosis and cardiac dysfunction (Tsuda et al., 2017). β-Adrenergic and Ang-II receptor antagonists are used to treat patients with chronic heart failure long-term. In the current sequencing study, a reduction of β1-adrenergic receptors was observed and may be associated with increased receptor internalization and recycling, which occurs through clathrin vesicles being degraded (Von Zastrow & Kobilka, 1992; Tolbert & Lameh, 1996; Zhang et al., 1998).

Studies have demonstrated that LP offspring showed enhanced glycemia and insulinemia after the glucose tolerance test and peripheral insulin resistance (Zambrano et al., 2006; Blesson et al., 2014; Blesson et al., 2016). The PI3K/AKT/mTOR signaling pathway regulates signal transduction and biological processes such as cell proliferation, apoptosis, protein synthesis, metabolism, and angiogenesis. Under normal conditions, insulin by IRS-1 tyrosine phosphorylation triggers a signaling cascade with a vasodilating and anti-apoptotic effect throughout IRS/PI3K/AKT/mTOR pathway (Gao et al., 2002). However, factors that lead to insulin resistance, such as TNFα, fatty acids, Cblb, and Crebbp, inhibit the IRS-1 down-regulation. This effect led to an inactivation of the PI3K/AKT and stimulated ERK/MAPK pathway, promoting cell hypertrophy (Brownsey et al., 1997; Sykiotis and Papavassiliou, 2001). Erhuma et al. (2007) demonstrated that prenatal exposure to a low-protein diet is a disordered regulation of lipid metabolism in the aging rat. The ERK/MAPK pathway is involved in cell growth control, and the IRS/PI3K pathway in insulin metabolism (Cheatham et al., 1994; Saltiel & Kahn, 2001; Araújo et al., 2005). As observed in the current study, we may not rule out that peripheral insulin resistance in LP offspring could be associated with significantly enhanced adiposity index compared to the NP group at 48 weeks, as observed by authors (Zambrano et al., 2006; Blesson et al., 2014; Blesson et al., 2016). However, the finding did not confirm at 62 weeks of age. The mRNA encoding the type-2 corticotrophin-releasing hormone receptor was significantly increased in LP offspring. This transmembrane protease activates the atrial natriuretic peptide, whose deficiency may contribute to the development of arterial hypertension and heart failure (Li et al., 2017). It has recently been observed that it is expressed in exacerbating chronic heart disease (Tsuda et al., 2017). Furthermore, the Corin protein transcript defects have also been observed in LP progeny. Here, an enhanced expression of the mRNA encoding PCSK6 (Proprotein convertase subtilisin/Kexin-6), a primary activator of Corin (Chen et al., 2015), was shown in 62-week-old LP offspring. However, even though the direct activator of Corin mRNA is increased in the present model, our data could not infer this protein's participation in the LP offspring's heart.

Once that prior study has demonstrated that gestational protein restriction leads to increased apoptosis of cardiomyocytes (Cheema et al., 2005), the present study may suggest that gene expression related to apoptosis and pro-autophagy observed here in 62-week-old LP offspring could lead to changes in cardiac functionality.

Pro-oncogenes are genes necessary for cellular homeostasis, responsible for the growth, proliferation, and survival mechanisms related to cancer presence and physiopathology (Hanahan & Weinberg, 2000). Therefore, the increase in the expression of the pro-oncogene genes could be related to cell growth in 62-week-old LP progeny. Simultaneously, several genes expression related to membrane components, Ca++ transport, ion channels, cytoskeleton, metabolism, and enzymatic functions were shown in this study. However, it is unclear to infer specific tasks since they have a variety of roles and participate in different biological processes.

A prior study in male mice submitted to gestational protein restriction (9%) has shown a mean survival lifetime of 69 weeks, compared to mothers that received a regular protein diet (18% casein), which showed an average lifetime survival of 74 weeks (Langley-Evans & Sculley, 2006). Based on these data, the age of 62 weeks for both progenies was determined as the deadline point for evaluating the heart structure, adipose tissue mass, mRNA sequence studies, and blotting data as defined in the experimental design.

The heart structural changes could be partly due to cardiac miRNA expression modulating several genes whose function is associated with cardiac morphogenesis and function. Many gene-encoding changes resulting from gestational protein restriction could be related to several visceral offspring disorders in adulthood compared to NP progeny. Those were defined either by metabolic or inflammatory activity and oxidative stress, as well as by apoptosis, autophagy, myocyte hypertrophy, interstitial fibrosis, and precocious cardiomyocyte senescence from programmed offspring. Taking into account together, they may lead to early changes in cardiac function, causing heart failure and advanced death (Figure 9). In conclusion, the deleterious cardiac repercussions in the 62-week-old LP compared to age-matched NP offspring could be related to the lower birth weight in programmed offspring, followed by the catch-up growth phenomenon.

Additionally, metabolic disorders that occur parallel to genes encoding expression may be involved in the altered adrenergic and renin-angiotensin-aldosterone compound system, oxidative stress, and inflammatory tissue deregulation. The above

disorders may lead to early cardiac hypertrophy, fibrosis, and senescence. The focus of the present study is on the heart. However, we could not rule out gene expression changes that could be secondary to adaptive responses to the programming to physiologic responses of the kidney, instead of the direct programming effect of LP diet on the male offspring heart sustained across the lifespan. Thus, a more dynamic analysis would be required to reach that conclusion; hypothetically, all these phenomena would promote organ dysfunction and the premature death of LP offspring (Figure 9).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Â NCBI's Gene Expression Omnibus, accessible through GEO Series accession number GSE188836 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188836).

Ethics statement

The animal study was reviewed and approved by The Institutional Ethics Committee reviewed and approved the experimental protocol (CEUA/Unicamp, protocol #4272-1, 2016) at the Campinas State University (Unicamp)—Campinas, SP, Brazil.

Author contributions

MSF: data curation, investigation, formal analysis, methodology, visualization, writing–original draft; ATF: methodology and visualization; SAV: methodology and supervision; JARG: funding acquisition, formal analysis, visualization, writing-review and editing; PAB: conceptualization, funding acquisition, formal analysis, methodology, supervision, visualization, writing-original draft and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.892322/full#supplementary-material

SUPPLEMENTARY FIGURE S1
The picture depicted the PCA plot to illustrate the relatedness of the samples used in RNA-Seq.

SUPPLEMENTARY FIGURE S2
The picture depicted volcano plot that represents the magnitude of changes in RNA-Seq.

SUPPLEMENTARY TABLE S1
Up (↑) and down (↓) regulated genes in left ventricles of low protein rats, biological function, and references.

SUPPLEMENTARY TABLE S2
The table depicted the values obtained for the genes studied and folded changes and adjusted p-values.

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Glossary

ADRB1 adrenoceptor beta 1

AngII angiotensin II

ANOVA variance analysis

AT1 abrangiotensin receptor type 1

AT2 angiotensin receptor type 2

BEX1 brain-expressed X-linked protein 1

BW body weight

CARP cardiac ankyrin repeat protein

Cblb casitas B-lineage lymphoma b

CEMIB Multidisciplinary Center for Biological Research in Science in Laboratory Animals.

abrCEUA Institutional Ethics Committee approved the experimental design on the Use of Animals

CONCEA Brazilian Council for Animal Experimentation

CREB cAMP-responsive element-binding

CREBBP CREB binding protein

CVD cardiovascular disease

Erk1/2 extracellular signal-regulated kinase1/2

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GPCR G protein-coupled receptor

IP6K3 inositol hexakisphosphate kinase-3

IRS1 insulin receptor substrate 1

abrIRS2 insulin receptor substrate 2

LaCTAD Central Laboratory for High-Performance Technologies

LHRI Laboratory of Human Retrovirology and Immunoinformatics

abrLP low-protein

LV left ventricle

MAPK mitogen-activated protein kinase

mTOR mammalian target of rapamycin

Na-K ATPase sodium pump

NOS1 nitric oxide synthases 1

NP normal protein

PCSK6 proprotein convertase subtilisin/Kexin-6

PI3K phosphoinositide 3-kinase

PKA protein kinase A

RAP1 a member of the RAS oncogene family

RAS renin-angiotensin-system

ROS: reactive oxygen species

RV right ventricle

SOD2 superoxide dismutase-2

TNF tumor necrosis factor

TXN2 thioredoxin 2

Wk week

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