MATERNAL DIET AND OFFSPRING HEALTH

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MATERNAL DIET AND OFFSPRING HEALTH

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EDITORIAL
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Editorial: Maternal Diet and Offspring Health

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Editorial on the Research Topic

Maternal Diet and Offspring Health

Maternal diet is recognized as a critical factor for shaping the life-long health of the next generation. It has been over three decades since the seminal work of Barker and colleagues demonstrated that a poor early start to life was associated with an increased risk for cardiovascular disease in adulthood (1). This Developmental Origins of Health and Disease (DOHaD) paradigm has expanded, and research shows that a wide range of nutrients and dietary patterns can influence offspring development (2). Significant advancements have been made in relation to the mechanisms which dictate adverse offspring health outcomes in response to maternal diet allowing for the development of novel early life strategies for the prevention of disease. However, this is still a developing topic and more insight into the biological mechanisms which underpin a healthy start to life are required to develop guidelines and strategies to prevent cardiometabolic disease early in life.

This Research Topic "Maternal Diet and Offspring Health" highlights the diversity of exposures and experimental models in the developmental programming field. This includes human cohort studies examining gestational weight gain on infant outcomes (Li et al.; Zong et al.), murine models of maternal undernutrition (Yi et al.; Zheng et al.), murine models of maternal high fat diet (HFD) intake during pregnancy (Bolam et al.; Buckels et al.; Wang et al.) and lactation (Hafner et al.) as well as the role of sweeteners [fructose and acesulfame-k (Ace-K)] during pregnancy (Bridge-Comer et al.) and agricultural reproductive studies (Luo et al.).

Identification of exposures that impact early life developmental outcomes continues to be a major DOHaD theme. Bridge-Comer et al. examined the impact of the caloric sweetener fructose and the artificial sweetener Ace-K during pregnancy and lactation on adult offspring in a mouse model. Male and female offspring had significant sex-specific differences across metabolic outcomes. Females, but not males, born to mothers who received Ace-K had reduced glucose tolerance, compared to fructose-exposed offspring. Both sexes displayed adipocyte hypertrophy when mothers were fed sweeteners and female offspring had dysregulated ovarian gene expression and estrus cycle disruption.

While many DOHaD studies focus on pre-clinical models, the role of DOHaD in relation to agriculture is often overlooked. Luo et al. demonstrated that fermented Radix puerariae residue, a traditional Chinese medicine, increased offspring weight at weaning, improved digestive efficiency and immune profiles as well as improving overall reproductive performance in pigs.

The work by Yi et al. and Zheng et al. further highlights the impact of offspring intake of a HFD as a "second hit" to exacerbate the negative effects of intrauterine undernutrition. Yi et al. reported on the effect of maternal smoking as a risk factor for fetal growth restriction and later cardiometabolic dysregulation. Offspring of pregnant mice exposed to cigarette smoke had increased adiposity and metabolic dysfunction, effects that were amplified in the setting

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of a postnatal HFD. Zheng et al. focused on a mouse model of maternal low-protein induced developmental programming. They examined the impact of a postnatal diet on hypothalamic modifications and showed that maternal protein restriction combined with postnatal HFD resulted in promotor region hypomethylation and increased expression of proopiomelanocortin (POMC) in the hypothalamus of male offspring. This was linked to impaired glucose function highlighting the importance of epigenetic processes in the manifestation of offspring health risks.

Although DOHaD is often associated with outcomes related to cardiometabolic disease, poor maternal nutrition during pregnancy and lactation can have a significant effect on multiple organ systems, including the musculoskeletal system. Buckels et al. summarize the impact of maternal HFD on bone microarchitecture in offspring highlighting the potential sex-specific mechanisms which are responsible for low bone mass and microarchitecture derangement. In addition, a study by Bolam et al. examines the role of maternal HFD on supraspinatus tendons of adult rat offspring. They demonstrate an increase in tendon elasticity in male but not female offspring associated with reduced gene expression of *Col1a1* (collagen type 1) and *Scx*, a transcription factor key for tendon formation. This is the first study to identify a role for maternal HFD in the biomechanical structure of offspring tendons.

The impact of the microbiome on peripheral metabolic organs has also recently been highlighted. Wang et al. show that a diet-induced maternal obesity model altered microbiome composition in mothers which was linked to fetal liver steatosis and placental structure. With increased incidence of children presenting with fatty liver disease, this study is important in deciphering the mechanisms which underlie fetal hepatic development and contribute to long-term metabolic disease. Many DOHaD studies focus on pregnancy although lactation also represents an important critical developmental window for the setting of metabolic cues in offspring. Hafner et al. examine this time period and demonstrated that a HFD during lactation resulted in fatty liver disease and insulin resistance in male but not female offspring, effects that were partially reversed with maternal metformin treatment.

Animal studies are essential for understanding the mechanisms that underly DOHaD. However, in order to be impactful these findings must be translated to a human setting. Li et al. examine the role of maternal dietary patterns and their impact of maternal gestational weight gain and offspring birth weight in the Tongji maternal and child health cohort. They show that a dietary pattern enriched in beans and vegetables is beneficial for preventing inappropriate gestational weight gain and ensuring healthy birthweights. Given variability in human populations, ensuring appropriate experimental power is essential for reliable results. Zong et al. examine a cohort of 9 million mother-infant pairs to show that pre-pregnancy BMI is an important factor for reducing the incidence of adverse birth outcomes.

This Research Topic demonstrates the diverse range of implications for maternal diet on offspring health. Novel exposures and new insights into the potential health risks have been identified in the studies presented. Further, the combination of pre-pregnancy through to lactational dietary modifications highlight the need to consider the role of each distinct timepoint as an opportunity for intervention to prevent the long-term health consequences of exposure to adverse maternal diets. Studies presented in this topic also uncover novel mechanisms which may help to design future interventions for both mothers and offspring.

AUTHOR CONTRIBUTIONS

CR wrote the editorial. MV edited the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Maternal High Fat Diet Consumption Exaggerates Metabolic Disorders in Mice With Cigarette-Smoking Induced Intrauterine Undernutrition

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Objectives: Maternal smoking causes fetal underdevelopment and results in births which are small for gestation age due to intrauterine undernutrition, leading to various metabolic disorders in adulthood. Furthermore, postnatal high fat diet (HFD) consumption is also a potent obesogenic factor, which can interact with maternal smoking. In this study, we aimed to determine whether maternal HFD consumption during pregnancy can reverse the adverse impact of maternal smoking and change the response to postnatal HFD consumption.

Methods: Female mice were exposed to cigarette smoke (SE, 2 cigarettes/day) or sham exposed for 5 weeks before mating, with half of the SE dams fed HFD (43% fat, SE+HFD). The same treatment continued throughout gestation and lactation. Male offspring from each maternal group were fed the same HFD or chow after weaning and sacrificed at 13 weeks.

Results: Maternal SE alone increased body weight and fat mass in HFD-fed offspring, while SE+HFD offspring showed the highest energy intake and glucose metabolic disorder in adulthood. In addition, postnatal HFD increased the body weight and aggravated the metabolic disorder caused by maternal SE and SE+HFD.

Conclusions: Maternal HFD consumption could not ameliorate the adverse effect of maternal SE but exaggerate metabolic disorders in adult offspring. Smoking cessation and a healthy diet are needed during pregnancy to optimize the health outcome in the offspring.

Keywords: smoke exposure, high fat diet, feeding regulation, glucose intolerance, myogenes

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WHAT THIS PAPER ADDS

What Is Already Known on This Subject

Maternal smoking is one of the known risk factors for the development of obesity in offspring. A number of studies have provided evidence that maternal cigarette smoke exposure (SE) leads to low birth weight and faster weight gain during the suckling period, called catch-up growth. This fast postnatal growth is commonly observed in children, which may lead to obesity during childhood as shown in the weaning offspring in this study.

What Important Gaps in Knowledge Exist on This Topic

Maternal high fat diet (HFD) consumption, on the other hand, can lead to *in utero* overnutrition. However, it is unclear whether in the setting of maternal SE, HFD consumption may mask the impact of *in utero* undernutrition caused by maternal SE. Furthermore, we cannot exclude the possibility that postnatal HFD consumption may worsen the regulation of energy consumption in offspring with *in utero* SE.

What This Paper Adds

Our study demonstrated that maternal SE during pregnancy results in increased adiposity and metabolic disorders if the offspring are exposed to HFD after weaning. The additional exposure to HFD failed to counteract with cigarette SE leading to even more severe metabolic disorders in adult offspring. Therefore, both quitting smoking and maintaining a healthy diet are vital for the healthy future of the offspring.

KEY MESSAGES

- Maternal smoking exposure (SE) decreased body weight without affecting the daily energy intake of breeders.
- Maternal HFD consumption could not ameliorate the adverse effect of maternal SE but exaggerate metabolic disorders in adult offspring.
- Postnatal HFD failed to counteract with cigarette SE but further increased the body weight and aggravated the metabolic disorder caused by maternal SE and SE+HFD.

INTRODUCTION

Obesity is occurring at alarming rates globally, which is linked to various complications, such as metabolic disorders, diabetes, cardiovascular disease, cancers, osteoarthritis, and reproductive problems (1–3). About 2.1 billion people worldwide are estimated to be overweight or obese, increasing their risk of developing associated insulin resistance and cardiovascular disease, adding to the already enormous cost of obesity-related diseases (3, 4). The consumption of food high in energy and fat is the major driver of the obesity pandemic. However, many obese people may also have had a suboptimal intrauterine environment which may interact with obesity-induced risks.

Maternal smoking is one of the known risk factors for the development of obesity in offspring. Epidemiological investigations revealed that maternal smoking/second-hand cigarette smoke exposure (SE) during pregnancy is also a major cause of intrauterine undernutrition, even the mothers do not necessarily eat less during pregnancy compared with non-smokers (5, 6). This is caused by placental limitation, leading to pre-term birth, low body weight, and reduced head circumference at birth (7–9). However, postnatal catch-up growth results in maternal smoking being associated with an increased risk of obesity in offspring in both childhood and adulthood (10–12). Secondary to this catch-up growth, maternal SE has also been shown to contribute to later metabolic disorders in the offspring, such as glucose intolerance and type 2 diabetes, fatty liver changes, dyslipidemia, and cardiovascular disease (13–17). This has been suggested to be linked to increased eating disorders in such offspring (8, 18). Therefore, the impact of maternal SE offspring is more than just intrauterine undernutrition and fetal growth restriction, with long-lasting effects on one's adulthood.

Maternal high fat diet (HFD) consumption, on the other hand, can lead to *in utero* overnutrition. However, it is unclear whether in the setting of maternal SE, HFD consumption may mask the impact of *in utero* undernutrition caused by maternal SE. Furthermore, we can't exclude the possibility that maternal HFD consumption may worsen the regulation of energy consumption in offspring with *in utero* SE, as both can encourage energy overconsumption in rodent models (19, 20). Maternal nicotine exposure is a strong risk factor for obesogenic overeating in childhood (21). Interestingly, prenatal growth retardation during infancy has been reported in obese mothers who smoked during pregnancy (5). This indicates that HFD consumption might not be able to reverse the intrauterine undernutrition resulted from maternal smoking. In animal studies, maternal HFD consumption during pregnancy also results in increased milk intake during the suckling period, and overfeeding during this period can have additive effects to further induce fat over accumulation resulting in metabolic disorder (22, 23). However, this conclusion is difficult to apply to humans due to the complexity of dietary and smoking behaviors between different individuals, as well as other external factors.

One of the widely studied appetite regulatory networks is in the hypothalamus, consisting of neurons expressing the appetite stimulator neuropeptide Y (NPY) and its counterpart, appetite suppressors α-melanocyte stimulating hormone (α-MSH) coded by proopiomelanocortin (POMC) (24). While maternal SE may induce smoking quitting type of rebound response of NPY in the offspring's brain (6), maternal obesity leads to a heightened response of NPY signaling after fasting (25). However, it is unclear how maternal SE and maternal HFD consumption interact to influence brain appetite regulators in the offspring. Skeletal muscle is a key metabolic organ for glucose metabolism. Both maternal HFD and SE exposure during pregnancy may affect muscle genesis, and thereafter metabolic function (26, 27). Myoblast determination protein 1 (Myod1) promotes transcriptional activation of Myogenin (Myog) during myogenesis (28). It is unknown how they are affected by prenatal and postnatal insults, which were examined in this study.

Furthermore, epidemiological studies have found that HFD consumed early in life is a risk factor for childhood weight gain and later adulthood obesity, accompanied by various metabolic dysfunction (29, 30). Thus, we hypothesized that post-weaning HFD exposure may further exaggerate metabolic disorders caused by maternal SE, whereas, additional HFD exposure in the SE mothers may ameliorate the adverse impact on the metabolic regulators in the offspring. In this study, we exposed the dams to cigarette smoke with/without access to a HFD and also offered the same HFD to half of the litter after weaning. We aimed to examine the interaction between maternal and postnatal environmental factors on the metabolic outcomes in the offspring, including body weight, organ weight, gene expression of metabolic markers in the hypothalamus and metabolic organs.

MATERIALS AND METHODS

Animals

According to the previous findings on the strain dependence of the response to SE (31), Balb/c mice were used for this study. Female mice breeders (aged 6 weeks) were housed at 20 ± 2°C in sterile micro-isolator cages and maintained on a 12:12 h light/dark cycle (lights on at 06:00 h). They were allowed a week to adapt to their new environment, with *ad libitum* access to standard rodent chow and water. The study was approved by the Animal Ethics Committee of the San Yet-sun University (number: SYSU-IACUC-2020-B0552).

Modeling of Maternal SE and HFD Feeding

After acclimatization, female breeders were randomly divided into three groups with similar average body weight: sham exposed fed a chow (CHOW+SHAM, representing a healthy control), chow-fed SE (CHOW+SE, representing smokers consuming a balanced diet), and HFD-fed SE (HFD+SE, representing smokers consuming a "junk" diet). For SE, animals were placed inside a perspex chamber (18 L) and exposed to the smoke produced by two cigarettes (Double Happiness; Tar: 8 mg; nicotine: 0.7 mg; CO: 10 mg), twice daily for 5 weeks before mating, during pregnancy and lactation as described in Chan et al. (32). The sham exposed animals were handled identically but were not exposed to cigarette smoke. Mice were fed either standard rodent chow (3.76 kcal/g, 16% energy as fat, 20% as protein, Research Diets, Inc., United States), or a pellet HFD (4.7 kcal/g, 43% energy as fat, 20% as protein, 35% as carbohydrate, Research Diets, Inc., United States. **Supplementary Table 1**) (33, 34). Body weight and 24-h caloric intake were measured once a week as previously described (14, 35). After 5 weeks of pre-conditioning, females were housed and mated with male mice. The same treatment was continued until pups weaned.

Postnatal Litter Size Adjustment and Post-Weaning HFD Feeding

On day 1 after birth, litters were adjusted to a size of 4–6 animals per litter (sex ratio 1:1) to minimize the impact of milk competition. At the age of 20 days (weaning age), male pups were used in the current study. Half of the male pups from the same litter were given a chow diet, while the other half were given the pellet HFD used in the dams. This further yielded six experimental groups, described as the maternal diet + maternal exposure – offspring diet. The six groups were CHOW+SHAM-CHOW, CHOW+SE-CHOW, HFD+SE-CHOW, CHOW+SHAM-HFD, CHOW+SE-HFD, and HFD+SE-HFD. Body weight and food intake were measured once a week until mice reached 13 weeks of age.

Offspring IP Glucose Tolerance Test (IPGTT)

Intraperitoneal glucose tolerance test (IPGTT) was carried out as previously described (13). Briefly, 11 weeks old male offspring were fasted for 5 h. Blood samples were collected from the tail tip to establish a baseline glucose level (T_0), which was measured again at 15, 30, 60, and 90 min after glucose injection (2 g/kg, ip). The area under the curve (AUC) was calculated for each mouse during IPGTT.

Sample Collection

Dams were culled 1 day after pups were weaned and male offspring were culled at 13 weeks. After overnight fasting and deep anesthesia (ketamine/xylazine 180/32 mg/kg), blood was collected by cardiac puncture and blood glucose was measured immediately (Accu-Chek glucose meter, Roche) in the dams. Plasma was stored at −20°C for insulin and triglyceride (TG) measurements. Then animals were killed by decapitation and the hypothalamus was micro-dissected. Brown adipose tissue (BAT), epididymal fat, retroperitoneal (Rp) fat, mesenteric fat was dissected and weighed, as well as organs (liver and heart) and skeletal muscle [soleus, extensor digitorum longus (EDL), and tibialis]. Rp fat, BAT, and skeletal muscle were kept providing further markers of substrate metabolism.

Plasma TG and Insulin Assays

Plasma TG was measured using glycerol standard (equivalent to 0–8.46 mM; G7793, Sigma-Aldrich) and TG reagent (T2449, Sigma-Aldrich) as previously described (35). Briefly, samples and standards were incubated with triglyceride reagent at 37°C for 20 min and read on a microplate reader (51119000, Thermo Scientific) at 490 nm. Plasma insulin concentrations were measured using a commercially available ELISA kit (KA3812, Abnova).

Quantitative Real-Time PCR

Total RNA was isolated using TriZol reagent (15596026, Invitrogen) according to the manufacturer's instructions. The purified total RNA was used as a template to generate first-strand cDNA synthesis kit (RR036A, Takara). TaqMan probe/primers that were pre-optimized and validated by the manufacturer (only probe sequence provided by Thermo Fisher Scientific, USA, **Supplementary Table 2**) were used for quantitative real-time PCR (StepOnePlus Real-Time PCR System, Applied Biosystem). Markers of appetite regulation, including Npy, Npy Y1 receptor (Npy1r), Pomc, and single minded gene (Sim1), were measured in the hypothalamus. Marker involved in substrate metabolism carnitine palmitoyl-transferase (Cpt1α) and Tnfα were measured

TABLE 1 | Parameters of the dams.

	Chow+Sham ($n = 11$)	Chow+SE ($n = 11$)	HFD+SE ($n = 10$)
BW initial (g)	18.47 ± 0.35	18.30 ± 0.40	18.01 ± 0.39
BW at mating (g)	20.47 ± 0.11	19.07 ± 0.18$^{\#}$	22.17 ± 0.58*
BW at weaning (g)	24.85 ± 0.98	20.59 ±0.24$^{\#}$	24.93 ± 0.98*
Energy intake (kJ/d)	36.82 ± 2.16	35.95 ± 1.96	41.97 ± 5.13
Liver (mg)	1,477.8 ± 33.9	1,168.1 ± 43.7$^{\#}$	1,289.5 ± 86.1*
Heart (mg)	119.6 ± 3.7	111.9 ± 2.4$^{\#}$	138.0 ± 7.0*
BAT (mg)	51.29 ± 4.2	48.58 ± 4.61$^{\#}$	79.81 ± 5.05*
Rp fat (mg)	32.11 ± 13.95	16.90 ± 8.46$^{\#}$	211.9 ± 32.9*
Epididymal fat (mg)	240.0 ± 87.3	122.6 ± 28.0$^{\#}$	811.8 ± 195.4*
Mesenteric fat (mg)	461.9 ± 50.4	379.9 ± 37.5$^{\#}$	628.8 ± 78.1*
Glucose (mM)	9.77 ± 0.11	9.43 ± 0.16$^{\#}$	9.13 ± 1.67

Results are showed as mean ± SEM. Data were analyzed by one-way ANOVA, followed by post-hoc LSD tests. $^{\#}p < 0.05$, maternal SE effect; $^{*}p < 0.05$, maternal HFD effect. BAT, brown adipose tissue; BW, body weight; Rp, retroperitoneal.

in the Rp fat. Thermogenesis markers Uncoupling protein (Ucp1 and Ucp3) were measured in BAT. Expression of muscle metabolic markers PPARγ coactivator (Pgc1α and Pgc1β), Myog and MyoD were measured in the soleus muscle.

Statistical Methods

Results are expressed as mean ± SEM. Data on blood glucose level change during IPGTT was analyzed using one-way analysis of variance (ANOVA) with repeated measures, followed by *post-hoc* Fisher's Least Significance Difference (LSD) tests. Differences in other parameters in the dams and offspring were analyzed using one-way ANOVA and two-way ANOVA, respectively, followed by *post-hoc* LSD tests if the data were normally distributed. If not, data were log transformed to achieve normality of distribution before they were analyzed.

RESULTS

SE Decreased Body Weight Without Affecting the Daily Energy Intake of Breeders

Before the start of the experiment, the average body weight of female mice was similar among the three groups. After 5 weeks of treatments, mice exposed to cigarette smoke showed significantly lower body weight than those with sham exposure ($p < 0.05$, **Table 1**), whereas HFD feeding increased the body weight of the mice with SE ($p < 0.05$, **Table 1**). This effect on body weight persisted until these breeders were sacrificed. Interestingly, SE did not affect the daily caloric intake, however, mice consumed 45.6% more calories if they were fed a HFD while exposed to cigarette smoke.

SE reduced liver and fat mass, with reduced blood glucose level ($p < 0.05$ vs. CHOW+SHAM, **Table 1**) which was consistent with the literature. HFD+SE dams also had increased liver and heart weight, as well as body fat such as BAT, Rp fat, epididymal fat, and mesenteric fat, while SE markedly reduced the weights of liver, heart, and fat tissues ($p < 0.05$ vs. CHOW+SE), with some even greater than the control mice, however, the glucose level was further reduced.

Postnatal HFD Increased the Body Weight and Aggravated the Metabolic Disorder Caused by Maternal SE and SE+HFD

At weaning (postnatal day 20), CHOW+SE and HFD+SE pups appeared to have bigger body weights as compared with those from control dams (CHOW+SHAM), indicating that both maternal HFD and SE might raise the risk of obesity in young mice (**Table 2**).

At 13 weeks, chow-fed offspring from CHOW+SE and HFD+SE dams showed similar body weight with only larger liver and heart in the SE-CHOW offspring. They also had a similar ability to clear blood glucose during IPGTT (**Figures 1A,B**), with similar plasma insulin and TG levels among the 3 chow-fed offspring groups (**Figures 1C,D**).

With postnatal exposure to a HFD, the body weight, body fat (BAT, RP, Epididymal, and Mesenteric fat) and skeletal muscle mass (EDL, soleus, and tibialis), were significantly greater in the offspring treatment. The mice from SE and SE+HFD dams showed a faster growth rate as compared with those from CHOW+SHAM dams ($p < 0.05$, CHOW+SHAM-HFD vs. CHOW+SE-HFD, CHOW+SE-HFD vs. HFD+SE-HFD, **Table 2**). A significant increase in energy intake was only observed in the HFD+SE-HFD offspring (**Table 2**). Moreover, CHOW+SE-HFD offspring showed significantly bigger heart and muscle weights, while HFD+SE-HFD offspring only showed larger muscle weight (EDL and Soleus) ($p < 0.05$, CHOW+SHAM-HFD vs. CHOW+SE-HFD, CHOW+SE-HFD vs. HFD+SE-HFD, **Table 2**). HFD+SE-HFD offspring developed more severe glucose intolerance during IPGTT than CHOW+SHAM-HFD and CHOW+SE-HFD offspring ($p < 0.05$, **Figure 1B**); however, only CHOW+SHAM-HFD and HFD+SE-HFD offspring showed increased plasma insulin levels (**Figure 1C**). In addition, plasma TG levels were not affected by postnatal HFD-consumption (**Figure 1D**).

Thus, maternal intervention, including SE and SE+HFD, resulted in increased body weight at weaning; however, this difference due to maternal programming diminished after consuming a balanced chow diet. While maternal SE increased body weight and heart weight without affecting adiposity when the offspring consumed a HFD, maternal exposure to both SE and HFD significantly increased caloric intake and resulted in the largest fat mass although without statistical significance.

Effects on Hypothalamic Appetite Regulators in Male Offspring

To investigate the effects of the maternal and postnatal HFD consumption on the feeding regulators, we checked the mRNA expression in the hypothalamus. In chow-fed offspring, *Pomc* mRNA expression was significantly up-regulated in the HFD+SE-CHOW group ($p < 0.05$ vs. CHOW+SHAM,

TABLE 2 | Parameters of the male offspring.

Maternal treatments	CHOW+SHAM	CHOW+SE	HFD+SE	CHOW+SHAM	CHOW+SE	HFD+SE
Offspring diet	CHOW			HFD		
	(*n* = 16)	(*n* = 8)	(*n* = 12)	(*n* = 16)	(*n* = 8)	(*n* = 13)
BW at 20 d (g)	7.01 ± 0.21	8.67 ± 0.33$^{\#}$	8.63 ± 0.30$^{\$}$	7.14 ± 0.24*	9.28 ± 0.31$^{*\#}$	8.99 ± 0.34$^{*\$}$
BW at 13 weeks (g)	19.71 ± 0.32	21.4 ± 0.27	20.59 ± 0.47	25.22 ± 0.66*	27.6 ± 0.64$^{*\#}$	26.99 ± 0.96*
Energy intake (kJ/d)	46.19 ± 8.69	46.55 ± 6.99	36.87 ± 4.81	58.51 ± 3.71	52.74 ± 2.82	64.54 ± 1.81*
Liver (mg)	879.9 ± 22.4	969.6 ± 15.2$^{\#}$	883.7 ± 18.5	1,055.1 ± 25.5*	1,093.1 ± 16.5*	1,149.8 ± 50.3*
Heart (mg)	101.3 ± 2.3	110.6 ± 3.3$^{\#}$	107.6 ± 3.6	125.0 ± 2.1*	137.8 ± 2.9$^{*\#}$	131.5 ± 2.49*
BAT (mg)	66.13 ± 1.26	68.4 ± 2.35	70.8 ± 2.45	118.9 ± 5.58*	124.0 ± 8.67*	134.6 ± 8.05*
Rp fat (mg)	125.4 ± 9.2	145.7 ± 13.8	170.3 ± 7.8	412.6 ± 38.1*	474.3 ± 51.4*	452.4 ± 39.8*
Epididymal fat (mg)	295.7 ± 20.0	366.0 ± 20.0	391.0 ± 19.9	906.8 ± 88.9*	1,101.5 ± 103.8*	1,189.8 ± 116.8*
Mesenteric fat (mg)	398.0 ± 18.2	460.8 ± 11.0	466.0 ± 27.6	592.6 ± 37.3*	642.2 ± 48.8*	704.5 ± 44.2*
EDL (mg)	18.70 ± 0.85	18.41 ± 0.53	17.73 ± 0.69	20.56 ± 0.46*	23.89 ± 0.95$^{*\#}$	21.29 ± 0.94$^{*\$}$
Soleus (mg)	10.05 ± 0.36	11.33 ± 0.56	10.81 ± 0.37	12.28 ± 0.28*	15.4 ± 0.79$^{*\#}$	12.26 ± 0.66$^{*\$}$
Tibialis (mg)	70.48 ± 1.58	75.58 ± 1.28	71.29 ± 2.19	80.98 ± 1.33*	88.66 ± 1.91$^{*\#}$	83.83 ± 2.41*

Results are presented as mean ± SEM. Data were analyzed by multi-factor ANOVA, followed by post-hoc LSD tests. $^{*}p < 0.05$, postnatal HFD effect; $^{\$}p < 0.05$, maternal HFD effect; and $^{\#}p < 0.05$, maternal SE effect. BAT, brown adipose tissue; BW, body weight; EDL, extensor digitorum longus; Rp, retroperitoneal.

Figure 2A), whereas *Sim 1* was significantly increased in the CHOW+SE-CHOW and HFD+SE-CHOW groups ($p < 0.05$ vs. CHOW+SHAM, **Figure 2B**). Moreover, hypothalamic *Npy* mRNA was only increased in CHOW+SE-CHOW offspring ($p < 0.05$ vs. CHOW+SHAM, **Figure 2C**), although the level in the HFD+SE-CHOW group was comparable to the CHOW+SE-CHOW group. However, *Npyr1r* mRNA was similar among chow-fed offspring.

In HFD-fed offspring, *Pomc* mRNA expression was increased by HFD consumption ($p < 0.05$ vs. CHOW+SHAM-CHOW, **Figure 2A**), which was further increased in CHOW+SE-HFD mice ($p < 0.05$, CHOW+SE-HFD vs. CHOW+SHAM-HFD). There was no difference in *Pomc* mRNA level between HFD+SE-HFD and CHOW+SE-HFD group. However, *Sim 1* was only significantly increased in the HFD+SE-HFD group compared with the CHOW+SHAM-HFD group ($p < 0.05$, **Figure 2B**). *Npy* was significantly upregulated in the CHOW+SE-HFD group ($p < 0.05$ vs. CHOW+SHAM-HFD, **Figure 2C**) although similar to its chow-fed littermates, whereas *Npyr1r* mRNA was significantly upregulated in the HFD+SE-HFD offspring ($p < 0.05$ vs. CHOW+SHAM-HFD, **Figure 2D**).

Effects on the Substrate Metabolic in the Fat and Muscle in the Offspring

Cpt1α is the rate-limiting step for fatty acid oxidation in the mitochondrial, while Tnfα is a pro-inflammatory cytokine which plays a key role in insulin resistance. The expression of *Cpt1α* and *Tnfα* were no affected by neither maternal nor postnatal interventions (**Figures 3A,B**). However, the expression of the thermogenesis markers *Ucp1* was significantly up-regulated by postnatal HFD consumption in CHOW+SHAM-HFD and HFD+SE-HFD groups compared with their chow-fed littermates ($p < 0.05$, **Figure 3C**), while *Ucp3* was significantly higher in the HFD+SE-HFD compared with CHOW+SHAM-HFD and CHOW+SE-HFD groups ($p < 0.05$, **Figure 3D**).

In soleus muscle, we check the expression of mitochondrial biogenesis markers Pgc1α and Pgc1β, which were not significantly affected by maternal programming nor postnatal HFD consumption (**Figures 4A,B**), albeit a trend decrease in *Pgc1β* expression in CHOW+SE-HFD and HFD+SE-HFD groups. Furthermore, *Myog* expression was not different among all groups (**Figure 4C**); however, *Myod1* expression was up-regulated in the HFD+SE-CHOW group ($p < 0.05$ vs. CHOW+SHAM-CHOW), which was further reduced by postnatal HFD feeding ($p < 0.05$ vs. HFD+SE-CHOW, **Figure 4D**).

DISCUSSION

In this study, we demonstrated that maternal SE exaggerates metabolic disorders if the offspring consume a HFD after weaning. The additional HFD consumption did not ameliorate or reverse the adverse effects due to maternal SE. On the contrary, maternal exposure to both SE and HFD led to the worst outcome in the offspring fed a HFD after weaning.

Exposure to cigarette smoke alone induced significant wasting in the dams, reflected by reduced both fat and lean body mass, consistent with a previous study (13). While additional HFD consumption only significantly increased the body weight at mating, after lactation, the body weight of HFD+SE dams was comparable to the control offspring. However, abdominal fat masses were increased in mice exposed to cigarette smoke at this time point. This is consistent with observations in humans that smoking encourages the development of central obesity albeit smaller body weight (36).

A number of studies have provided evidence that maternal SE led to low birth weight and faster weight gain during the suckling

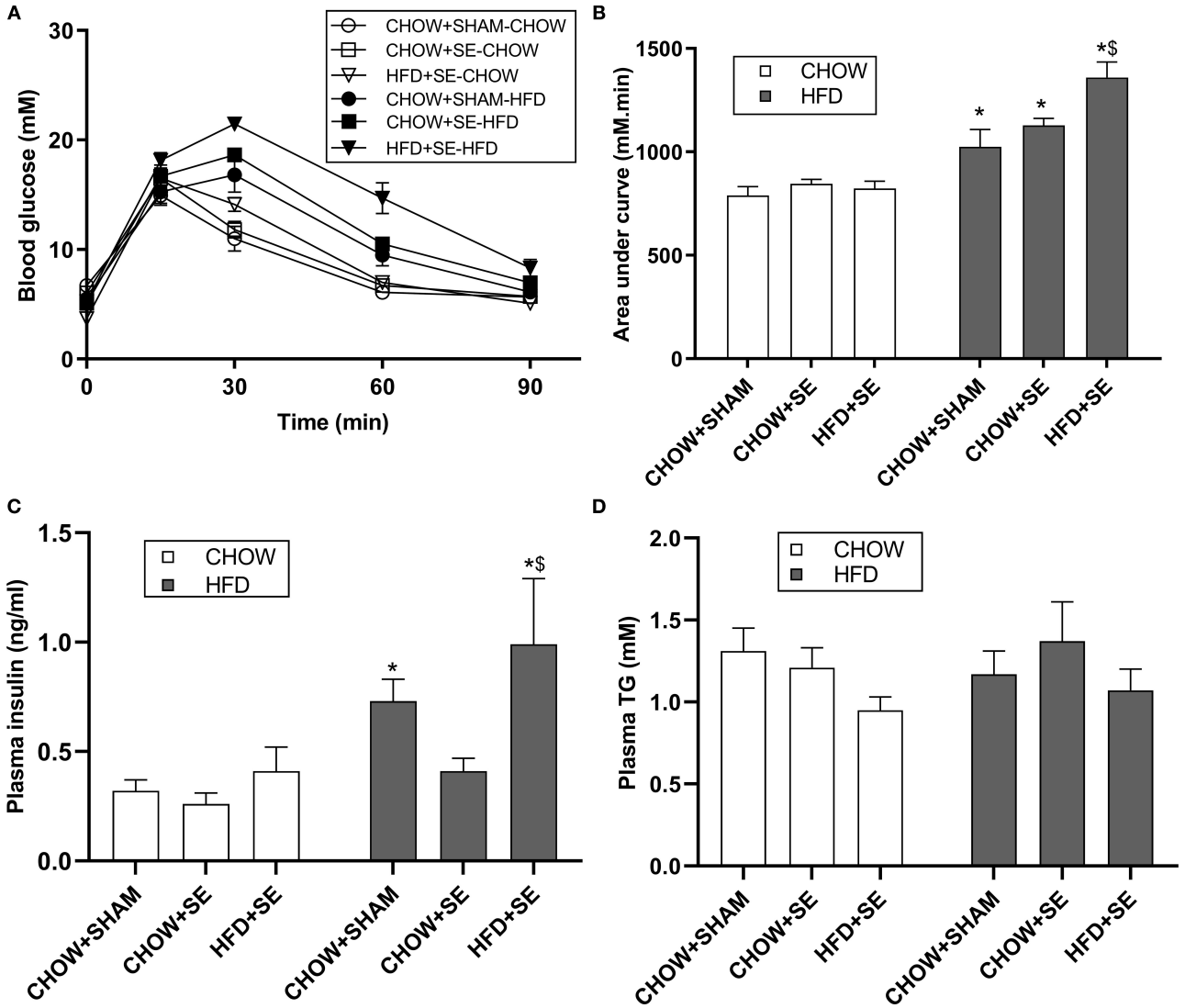

FIGURE 1 | Blood glucose and lipid profile in male offspring. **(A)** Change in blood glucose levels during an IPGTT at 11 weeks. The area under the curve for panel **(A)** is shown in panel **(B)**. **(C,D)** The concentration of insulin **(C)** and TG **(D)** in plasma in male offspring at 13 weeks. All statistical results are showed as mean ± SEM. Data were analyzed by two-way ANOVA, followed by *post-hoc* LSD tests. $^{*}p < 0.05$ postnatal HFD effect, $^{\$}p < 0.05$ maternal HFD effects.

period, called catch-up growth (6, 37, 38). This fast postnatal growth is commonly observed in children, which may lead to obesity during childhood as shown in the weaning offspring in this study (39, 40). Childhood BMI can positively correlate with that in adulthood, which has been well-represented in the offspring with *in utero* SE. This is not caused by overeating, as the caloric intake was similar between CHOW+SE-HFD and CHOW+SHAM-HFD offspring. Maternal nicotine exposure has been shown to reduce hypothalamic *Npy* and increase *Pomc* expression in newborns (6). The increase in both in the CHOW+SE-HFD offspring may reflect a withdrawn rebound, similar to the response in the smokers after quitting. This may be attributed to larger fat mass in CHOW+SE-HFD offspring. There are several homologs of UCPs, including UCP1 and UCP3, which are responsible for mediating thermogenesis and basal metabolic rate in fat (41). Previously study showed that caloric restriction could down-regulate UCP1 to and UCP3 reserve energy expenditure (42, 43). Thus, the HFD leads to an adaptive upregulation of UCPs to increase energy expenditure as shown in CHOW+SHAM-HFD mice. Compared with CHOW+SHAM-HFD offspring, those with intrauterine SE had suppressed thermogenesis marker UCP1 in their BAT, which may impair the adaptive increase in heat production observed in CHOW+SHAM-HFD offspring, resulting in increased fat mass in CHOW+SE-HFD offspring. Glucose intolerance in CHOW+SE-HFD mice may be due to insulin insufficiency, rather than insulin resistance in CHOW+SHAM-HFD, as reflected by plasma insulin levels in these two groups. Increased

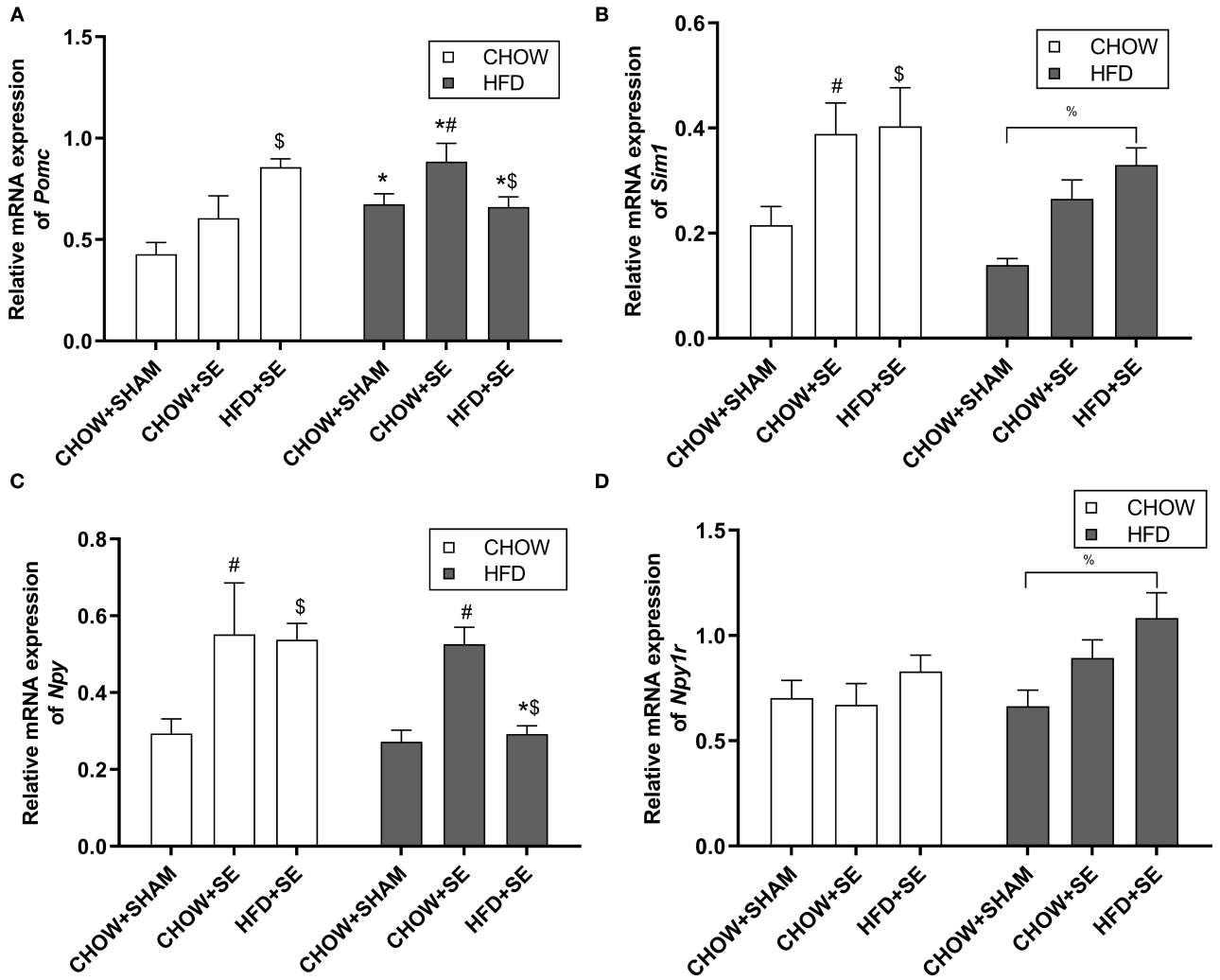

FIGURE 2 | Expression of the energy homeostatic regulator in the hypothalamus. Hypothalamic mRNA expression of *Pomc* **(A)**, *Sim1* **(B)**, *Npy* **(C)**, and *Npy1r* **(D)** in the male offspring at 13 weeks. Results are expressed as mean ± SEM. Data were analyzed by two-way ANOVA, followed by *post-hoc* LSD tests. $^{*}p < 0.05$, postnatal HFD effect; $^{\$}p < 0.05$, maternal HFD effect; and $^{\#}p < 0.05$, maternal SE effect. $^{\%}p < 0.05$.

insulin level is a sign of insulin resistance. HFD has been shown to reduce insulin sensitivity and promote the development of type 2 diabetes (44). Previous studies have shown that maternal nicotine treatment can interrupt β-cell functions in the offspring (45–47).

"Eat for two" is a traditional practice for pregnant women even in the current obesity pandemic. As intrauterine undernutrition is a key to maternal smoking, such practice may be more appealing. However, in this study, we have shown that the combination of maternal HFD and SE further exaggerates the metabolic disorders than maternal SE alone when the offspring were exposed to an obesogenic environment after birth. There are several mechanisms suggested in this study. Firstly, overeating was only observed in HFD+SE-HFD offspring, which may be driven by markedly upregulated Npy1r, the orexigenic receptor for NPY. This increase in activity was not counteracted by the adaptive increase in Sim1 expression which lies downstream of the receptor of the anorexigenic peptide αMSH. Secondly, the heightened glucose intolerance may be a combination of impaired β-cell function due to maternal SE and insulin resistance due to both intrauterine and postnatal HFD exposure. Albeit overeating, the body weight and fat mass in HFD+SE-HFD seem to be controlled. This may be due to more than doubled Ucp3 expression to increase the energy expenditure while energy was over consumed.

During the weight gain by HFD consumption, muscle mass is also increased to support the increased body weight, as shown in HFD-fed mice in this study. The greatest increase in HFD+SE-HFD mice may be proportional to their body weight. Pgc1α controls mitochondrial biogenesis and angiogenesis in skeleton muscle (48–50); whereas, Pgc1β activates an anti-angiogenesis gene program in the skeletal muscle and its overexpression can induce muscle wasting by inhibiting ubiquitin-mediated proteolysis (51, 52). In our present study, the trending decrease in Pgc1β in mice from SE dams can help to explain the

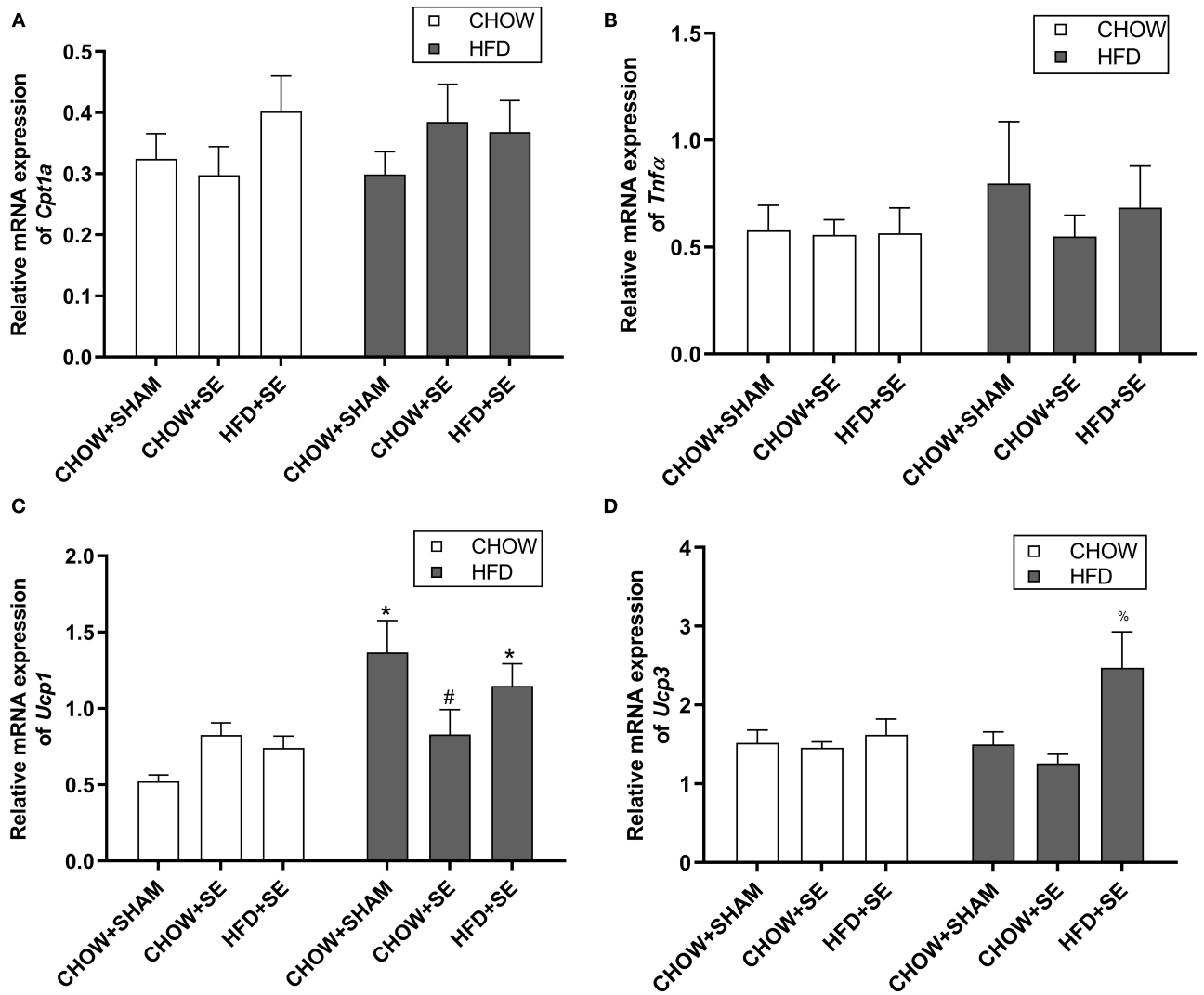

FIGURE 3 | Expression of energy metabolic regulators in fat tissue. mRNA expression of *Cpt1α* **(A)**, *Tnfα* **(B)** in the white fat and, *Ucp1* **(C)**, and *Ucp3* **(D)** in the brown fat in the male offspring 13 weeks. Results are expressed as mean ± SEM. Data were analyzed by two-way ANOVA, followed by *post-hoc* LSD tests. $^{*}p < 0.05$, postnatal HFD effect and $^{\#}p < 0.05$, maternal SE effect. $^{\%}p < 0.05$ vs. CHOW+SHAM-HFD and CHOW+SE-HFD.

increased muscle mass. However, this did not seem to lead to an improvement in muscle metabolic function. Previous studies suggested that Myod1 could promote transcriptional activation to regulate the expression of muscle-specific genes, including Myog, which plays a crucial role in the terminal differentiation during myogenesis (28, 53). In this study, we found significantly altered *Myod1* expression but not *Myog* by maternal HFD+SE. This may be an adaptation to prevent a reduction in muscle mass by maternal SE, which is common in smokers (54). However, the Myod1 changes in offspring from HFD+SE dams was reversed by postnatal HFD consumption. This may be due to myogenesis, as we observed in the other groups fed a HFD. However, further studies are needed to examine the metabolic functions of the muscle as well as mitochondrial function which is beyond the scope of this study.

Whilst there was increased fat mass and unchanged fatty acid metabolic marker Cpt1α, plasma TG was not affected by maternal programming nor postnatal HFD consumption, which might be due to the mouse strain specific. It has previously been shown that with cigarette SE only during lactation, there were decreased proteins levels of Ucp1 and Cpt1 and reduced sympathetic nerve stimulation upon BAT in female adult rat (55). The same was observed with the administration of isolated nicotine through minipumps on the dams at the same period (56). Similarly, in other rat model of SE only during lactation, SE group male adult offspring did not changed insulinemia, despite higher serum glucose levels, suggesting a pancreatic insulin secretory failure (57). Nevertheless, it needs to be noted that none of the above metabolic abnormalities were observed in the chow-fed offspring. This highlights the importance of a healthy diet to prevent the

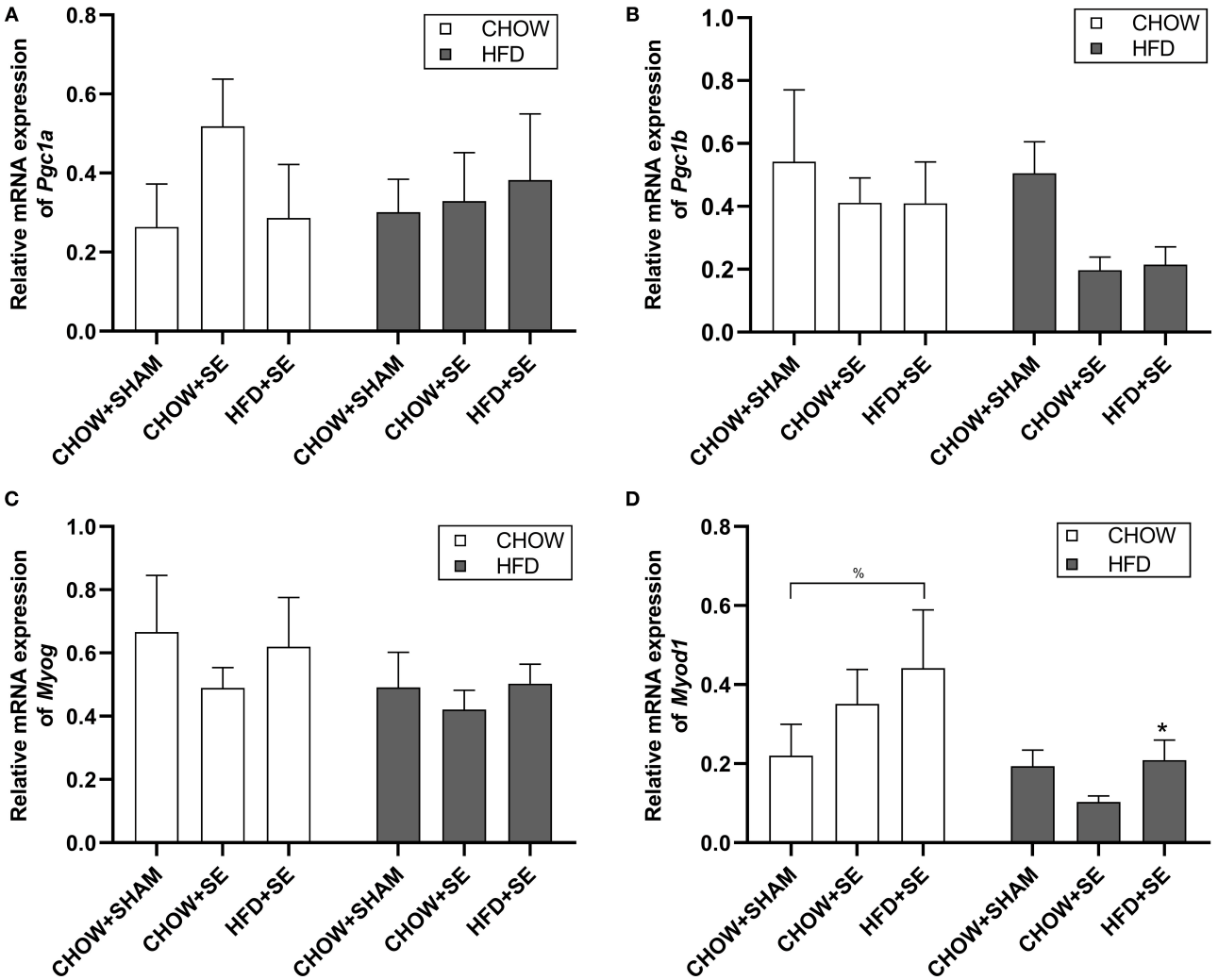

FIGURE 4 | Expression of the metabolic regulator and myogenesis related gene in skeletal muscle tissue. Muscle mRNA expression of *Pgc1α* **(A)**, *Pgc1β* **(B)**, *Myog* **(C)**, and *Myod1* **(D)** in the male offspring at 13 weeks. Results are expressed as mean ± SEM. Data were analyzed by two-way ANOVA, followed by *post-hoc* LSD tests. $^{*}p < 0.05$, postnatal HFD effect; $^{\%}p < 0.05$.

adverse impact of maternal programming on metabolic outcomes in adulthood. As this study was only performed with male mice, it cannot be directly extended to the female offspring, due to the sexual differences during maternal programming as shown by the others (13, 58). In a similar model of SE, females offspring showed increased glucose tolerance by maternal SE, which is consistent with our founding (13). In addition, another study showed that female offspring from the SE dams had lower levels of anorexigenic neuropeptides, Cocaine-, and amphetamine-regulated transcript and MSH, in their brains (59).

In conclusion, this study demonstrated that maternal SE during pregnancy results in increased adiposity and worsened metabolic disorders if the offspring are exposed to HFD after weaning. The additional maternal exposure to HFD interacts with SE which exacerbated metabolic disorders in the male offspring by disrupting metabolic regulators. Therefore, both quitting smoking and maintaining a healthy diet are vital for the healthy future of the offspring.

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 Institutional of Animal Care and Committee of San Yet-sun University.

AUTHOR CONTRIBUTIONS

TH: conceptualization, methodology, visualization, investigation, and writing – original draft. MY and YZ: investigation and editing. XH: investigation. NW: visualization and investigation. YC, PL, and JY: supervision and validation. CC and BO: supervision and review. CY: supervision, writing,

review, and editing. All authors contributed to the article and approved the submitted 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/fnut.2021.638576/full#supplementary-material

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Long-Term Effects of Maternal Low-Protein Diet and Post-weaning High-Fat Feeding on Glucose Metabolism and Hypothalamic POMC Promoter Methylation in Offspring Mice

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Substantial evidence indicated that maternal malnutrition could increase the susceptibility to obesity, insulin resistance, and type 2 diabetes in adulthood. It is increasingly apparent that the brain, especially the hypothalamus, plays a critical role in glucose homeostasis. However, little information is known about the mechanisms linking maternal protein restriction combined with post-weaning high-fat (HF) feeding with altered expression of brain neurotransmitters, and investigations into the epigenetic modifications of hypothalamus in offspring have not been fully elucidated. Our objective was to explore the effects of maternal protein restriction combined with post-weaning HF feeding on glucose metabolism and hypothalamic POMC methylation in male offspring mice. C57/BL6 mice were fed on either low-protein (LP) or normal chow (NC) diet throughout gestation and lactation. Then, the male offspring were randomly weaned to either NC or high-fat (HF) diet until 32 weeks of age. Gene expressions and DNA methylation of hypothalamic proopiomelanocortin (POMC) and melanocortin receptor 4 (MC4R) were determined in male offspring. The results showed that birth weights and body weights at weaning were both significantly lower in male offspring mice of the dams fed with a LP diet. Maternal protein restriction combined with post-weaning high-fat feeding, predisposes higher body weight, persistent glucose intolerance (from weaning to 32 weeks of age), hyperinsulinemia, and hyperleptinemia in male offspring mice. POMC and MC4R expressions were significantly increased in offspring mice fed with maternal LP and postnatal high-fat diet ($P < 0.05$). Furthermore, maternal protein restriction combined with post-weaning high-fat feeding induced hypomethylation of POMC promoter in the hypothalamus ($P < 0.05$) and POMC-specific methylation (%) was negatively correlated with the glucose response to a glucose load in male offspring mice ($r = -0.42$, $P = 0.039$). In conclusion, maternal LP diet combined with post-weaning high-fat feeding

predisposed the male offspring to impaired glucose metabolism and hypothalamic POMC hypomethylation. These findings can advance our thinking about hypothalamic POMC gene methylation between maternal LP diet combined with post-weaning high-fat feeding and metabolic health in offspring.

Keywords: glucose metabolism, hypothalamus, DNA methylation, offspring, maternal low-protein diet, post-weaning high-fat feeding

INTRODUCTION

Maternal malnutrition has been associated with the onset of metabolic diseases in adulthood, including obesity, insulin resistance, and diabetes (1–4). It has been proposed to result from unbalanced dietary patterns during pregnancy and after weaning. Numerous animal experiments, including our previous studies, have indicated that maternal low-protein (LP) diet combined with a post-weaning high-fat (HF) diet can significantly increase susceptibility to obesity, impaired glucose tolerance, and insulin resistance in offspring (1, 2, 5).

However, the mechanisms underlying maternal and postnatal unbalanced diets and metabolic diseases in adulthood have not been fully elucidated. It has been widely accepted that epigenetic modifications may be the underlying mechanisms of these effects, which may link such imbalanced nutrition with the risks of metabolic diseases (6–8). It is reported that most peripheral organs, including the liver, pancreas, skeletal muscle, and adipose tissue appeared to be imprinted by unbalanced nutrition, which can be associated with epigenetic modulation of key developmental gene expressions (9, 10). Hypermethylation of these CpG islands has a specific effect on repressing transcription, whereas hypomethylation of CpG islands is related to transcriptional activation. When a CpG island in the promoter region of a gene is methylated, expression of the gene is repressed and *vice versa*. Our previous study showed that maternal LP diet can program glucose metabolism and hepatic microRNA expressions in early life offspring (11).

Recently, it became increasingly apparent that the brain, especially the hypothalamus, is the control center for energy homeostasis (12). Anorexigenic neuropeptides, located in the mediobasal hypothalamus, such as proopiomelanocortin (POMC), and melanocortin-4 receptor (MC4R), mediate satiety and increase energy expenditure, thus lead to loss of weight (13–15). Several studies have focused on the effects of maternal over-nutrition on hypothalamic neuropeptides, with increased expressions of POMC and MC4R in offspring (16–18). However, little information is known about the mechanisms linking prenatal LP and postnatal HF diets with altered expression of brain neurotransmitters, and investigations into the epigenetic modification of hypothalamus in offspring are limited. Our objective was to determine the programming effects of maternal protein restriction combined with post-weaning HF feeding on mice offspring, including metabolic health, hypothalamic neuropeptide gene expressions, and hypothalamic POMC gene methylation.

MATERIALS AND METHODS

Ethical Statement

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, and procedures were approved by the Peking University First Hospital Institutional Animal Care and Use Committee.

Experimental Design and Animal Model

Female C57BL/6J mice were maintained under controlled conditions and randomly assigned to either a LP diet (8% protein) or normal chow (NC) diet (20% protein) during pregnancy and lactation, as we previously described (5). Nutritional composition of the diets is shown in **Supplementary Table 1**. In order to avoid nutritional bias among litters, litter size was standardized to six pups. At 3 weeks of age, offspring mice were weaned either an HF diet (HF: 58% kcal fat) or an NC diet. Thus, it generated four groups of offspring mice: NC–NC, LP–NC, NC–HF, LP–HF (n = 8–10/group) (Abbreviations denoted before and after the dash line were as dam and offspring diets, respectively).

Birth weight of newborn mice and body weight at weaning were measured. Weight gain and food intake in offspring mice were recorded periodically. All the offspring mice were anesthetized and sacrificed at 32 weeks of age. Schematic representation of the experimental feeding course was shown in **Supplementary Figure 1**. Blood samples were collected from the retrobulbar, intraorbital, capillary plexus in anesthetized mice, which were fasted 10-h. The hypothalamuses were dissected, snap frozen, and stored at −80°C for further analysis, as we previously described (19). In this study, we mainly focused on male offspring to prevent confounding factors related to the estrus cycle and hormone profile of female offspring. In addition, a sexually dimorphic manner has been reported in the maternal LP diet rodent model, which was not the concern of this study (20, 21).

Intraperitoneal Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (ipGTTs) were performed as previously described (22). Mice were fasted overnight (12 h) and injected intraperitoneally with glucose (2 g/kg body weight). Blood glucose concentrations were determined using a glucometer (Contour TS, Bayer, Beijing, P. R. China) and blood from the tail at baseline and 30-, 60-, and 120-min after glucose injection. Area under the curve (AUC) was calculated using the trapezoid method to evaluate blood glucose response to the ipGTTs.

Serum Hormone Measurements

Serum insulin was detected using the Mouse Ultrasensitive Insulin ELISA kit (ALPCO Diagnostics, Salem, NH, USA) and serum leptin was measured using the mouse ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the instructions of the manufacturers. Each sample was measured in duplicate.

RNA Extraction and RT-qPCR Analyses

RNA was extracted from hypothalamus using TRIzol reagent (Life Technologies Inc., Carlsbad, CA, USA) and 1 μg RNA was converted into cDNA by a reverse transcription procedure using the Power cDNA Synthesis kit (Promega BioSciences LLC, Sunnyvale, CA, USA), according to the protocol of the manufacturer. Then, cDNA was amplified using the appropriate primers and probes. The sequences of the primers are as follows: POMC: forward 5′-CGACAGGC AGGAGACTGAAC-3′, reverse 5′-CGCAGAGAAACGAGGGT TTG-3′; MC4R: forward 5′-TGAACTTCTGAGAGGCTGCG-3′, reverse 5′-TTCTCGGTTGACCAGTCTGC-3′; and β-actin: forward 5′-TGTTACCAACTGGGACGACA-3′, reverse 5′-GGG GTGTTGAAGGTCTCAAA-3′. Real-time PCR was performed and accurately measured using a standard TaqMan PCR kit protocol on an ABI prism Vii7 Sequence Detection System (ABI Prism® Vii7, Applied Biosystems, Life Technologies). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to the expression of the β-actin housekeeping gene (23). All reactions were carried out with three biological replicates, and each analysis consisted of three technical replicates.

POMC and MC4R Methylation Levels by Bisulfite Sequencing PCR

POMC and MC4R promoters methylation levels were examined by bisulfite sequencing PCR, as our previous study described (19). Precisely, genomic DNA was extracted from hypothalamus tissues in offspring mice, using an E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA, United States), and DNA samples were treated with sodium bisulfite, using EZ DNA Methylation Kit (Zymo Research, HiSS Diagnostics, Germany), according to the instructions of the manufacturer. POMC and MC4R promoter areas were amplified using the following primers: POMC: forward 5′-GATTGGTTTTTGGGG AGATTT-3′, reverse 5′-ATTTCAAAACCTTAAACAATTCCC T-3′; MC4R: forward 5′-TTTAAAATTTGGAAAGGAAAATTT-3′, reverse 5′-TACTAAAAACAAAATCAAAAACAAC-3′; and β-actin: forward 5′-TGTTACCAACTGGGACGACA-3′, reverse 5′-GGGGTGTTGAAGGTCTCAAA-3′. PCR amplification of genomic fragment of POMC and MC4R promoters was performed using BIOTAQ DNA Polymerase (Bioline USA Inc, Taunton, MA, United States). The PCR products were separated on 1.5% agarose gel followed by gel extraction with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and cloned into PGEMT-easy vectors (Promega, Madison, WI, United States). PGEMT-easy vectors were multiplied using JM109 competent *Escherichia coli* cells using standard procedures and then purified from the bacteria with QIAprep Spin Miniprep Kit (QIAGEN). At least 20 positive bacterial clones were conducted in each sample and a minimum of 95% bisulfite conversion was included in subsequent analyses. Figure generation, sequence analysis, and quality control were performed using BiQ Analyzer software.

Statistical Analysis

Data are presented as mean values ± SEM. Statistical analysis was conducted through analyses of variance (ANOVAs), with repeated measures where applicable. Bonferroni *post hoc* tests were performed to identify where statistically significant differences existed when ANOVAs were significant. Group differences in fasting blood glucose, serum hormone measurements, mRNA expression levels, and DNA methylation levels were analyzed by one-way ANOVA. Body weight and ipGTT were analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. Statistical significance was reached at a $P < 0.05$.

RESULTS

Effects of Maternal Protein Restriction and Post-weaning High-Fat Feeding on Birth Weight, Body Weight, and Food Intake in Offspring

Maternal LP diet during pregnancy and lactation induced lower birth weight in newborn mice ($P < 0.05$) (**Figure 1A1**). At weaning, body weight remained significantly decreased in mice offspring of dams fed with LP diet ($P < 0.01$) (**Figure 1A2**). At 8 weeks of age, no difference was found in body weight among the four groups. However, maternal LP diet combined with post-weaning HF-fed mice (LP–HF group) had increased body weight from 16-weeks of age until 32-weeks of age when mice were sacrificed, compared with both NC–NC and LP–NC groups (*P*-value as denoted) (**Figure 1B**). There was no difference in food consumption among offspring mice throughout the experiment (**Figure 1C**).

Long-Term Effects of Maternal Protein Restriction and Post-weaning High-Fat Feeding on Fasted Blood Glucose and Glucose Tolerance in Offspring From 8 to 32 Weeks of Age

As shown in **Figure 2** and **Supplementary Figure 2**, maternal protein restriction combined with post-weaning HF feeding (LP–HF) had impaired glucose tolerance from 8 weeks of age. At 8 weeks of age, fasted blood glucose concentration ($P < 0.05$) and blood glucose levels of the male offspring in the LP–HF group were significantly higher at 30 min ($P < 0.05$) after intraperitoneal glucose administration. However, there is no difference in AUC among the four groups. Then the glucose metabolism disturbance was exacerbated in the offspring of LP–HF group during the period from 8 to 32 weeks. At 32 weeks of age, fasted blood glucose

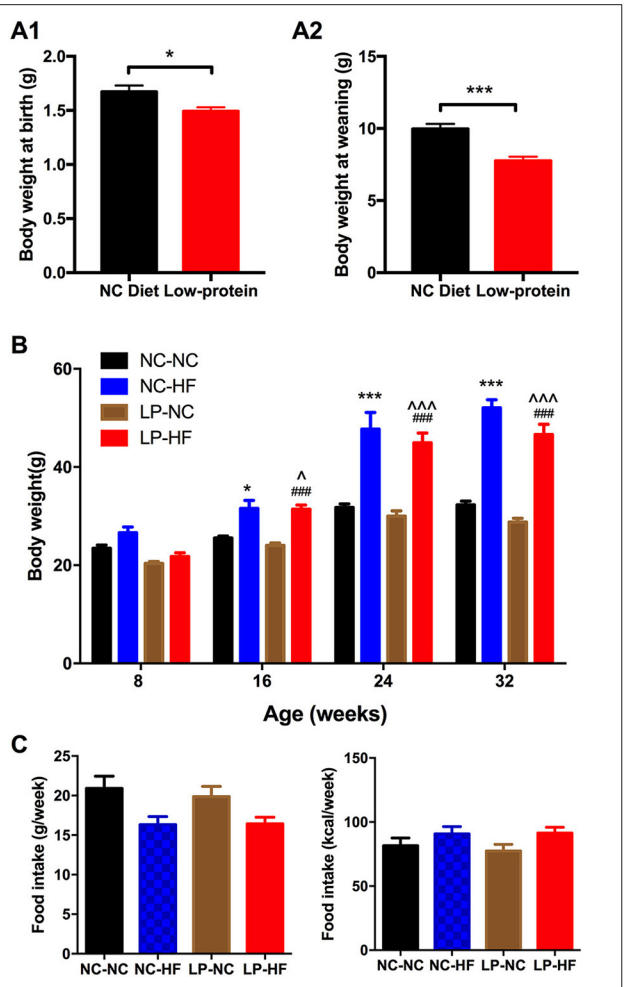

FIGURE 1 | Effects of maternal protein restriction and post-weaning high-fat feeding on body weight and food intake in offspring. **(A)** Birth weight; **(B)** body weight at weaning; **(C)** body weight from 8 to 32 weeks of age. Data were represented as mean ± SEM (n = 6–8/group). $^{*}P < 0.05$, $^{***}P < 0.001$ NC–HF *vs.* NC–NC group. $^{\#\#\#}P < 0.001$ LP–HF *vs.* LP–NC group. $^{\wedge}P < 0.05$, $^{\wedge\wedge\wedge}P < 0.001$ LP–HF *vs.* NC–NC group. Diet abbreviations: NC, normal chow; LP, low protein; HF, high fat. Dam and pup diets denoted before and after the dash line, respectively.

concentration was significantly increased in offspring mice exposed to maternal protein restriction combined with post-weaning HF feeding, compared with NC–NC ($P < 0.05$) and LP–NC ($P < 0.001$) groups, respectively. The blood glucose levels of the male offspring in the NC–HF and LP–HF groups were significantly higher at 30 min ($P < 0.001$), 60 min ($P < 0.001$), and 120 min ($P < 0.001$) after intraperitoneal glucose administration, compared with those of the NC–NC offspring. Consistently, the AUC of ipGTT was significantly greater in NC–HF and LP–HF than NC–NC offspring ($P < 0.001$). Thus, it indicates that maternal protein restriction combined with post-weaning HF feeding, predisposes persistent glucose intolerance in offspring mice from weaning to 32 weeks of age.

Maternal Protein Restriction and Post-weaning High-Fat Feeding Resulted in Hyperinsulinemia and Hyperleptinemia in Offspring Mice

Serum insulin concentration was significantly increased in offspring mice fed an HF diet whose mothers had been fed an LP diet, compared with NC–NC and LP–NC groups (both $P < 0.05$) (**Figure 3A**). We further detected serum leptin level in offspring mice. As a critical peripheral hormone, leptin can act on leptin receptors located in the arcuate nucleus of the hypothalamus to regulate appetite and energy homeostasis. Leptin levels were significantly higher in HF-fed offspring whose mothers had eaten the LP diet, compared with all the other offspring mice ($P < 0.001$) (**Figure 3B**).

Maternal Protein Restriction and Post-weaning High-Fat Feeding Regulated Hypothalamic POMC and MC4R Expressions in Offspring

To further assess the potential effects on the neuroendocrine control of body weight and energy homeostasis, we examined POMC and MC4R gene expression in the hypothalamus of the offspring. POMC and MC4R expressions were significantly increased in offspring mice exposed to maternal protein restriction combined with post-weaning HF feeding (both $P < 0.05$) (**Figures 4A,B**).

Effects of Maternal Protein Restriction and Post-weaning High-Fat Feeding on POMC Methylation in Offspring

Then, as one important epigenetic modification, DNA methylation levels of POMC and MC4R genes were further examined using MassARRAY EpiTYPER assays. We mainly concentrated on the CpG islands of POMC and MC4R gene promoters. We use online EMBOSS Cpgplot software to predict CpG island (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/). According to the prediction results, POMC gene has one CpG island with 24 CpG sites; however, no CpG islands were predicted of MC4R gene promoter. For POMC, methylation level was decreased at the specific sites of 5–7, 9–10, and 11–13 (all $P < 0.05$) (**Figure 5A**). The average methylation level of POMC promoter was significantly decreased in the HF-fed offspring whose mothers were fed on an LP diet ($P < 0.05$) (**Figure 5B**).

Correlation Between POMC-Specific Methylation and Glucose Metabolism in Offspring Mice

To further evaluate whether differential POMC-specific methylation was responsible for impaired glucose metabolism due to maternal LP and postnatal HF diet in offspring mice, Spearman's correlation analyses were performed between POMC-specific methylation (%) and fasted blood glucose (10-h fasting before sacrifice) and AUC of ipGTT, respectively. No association was observed between POMC-specific methylation

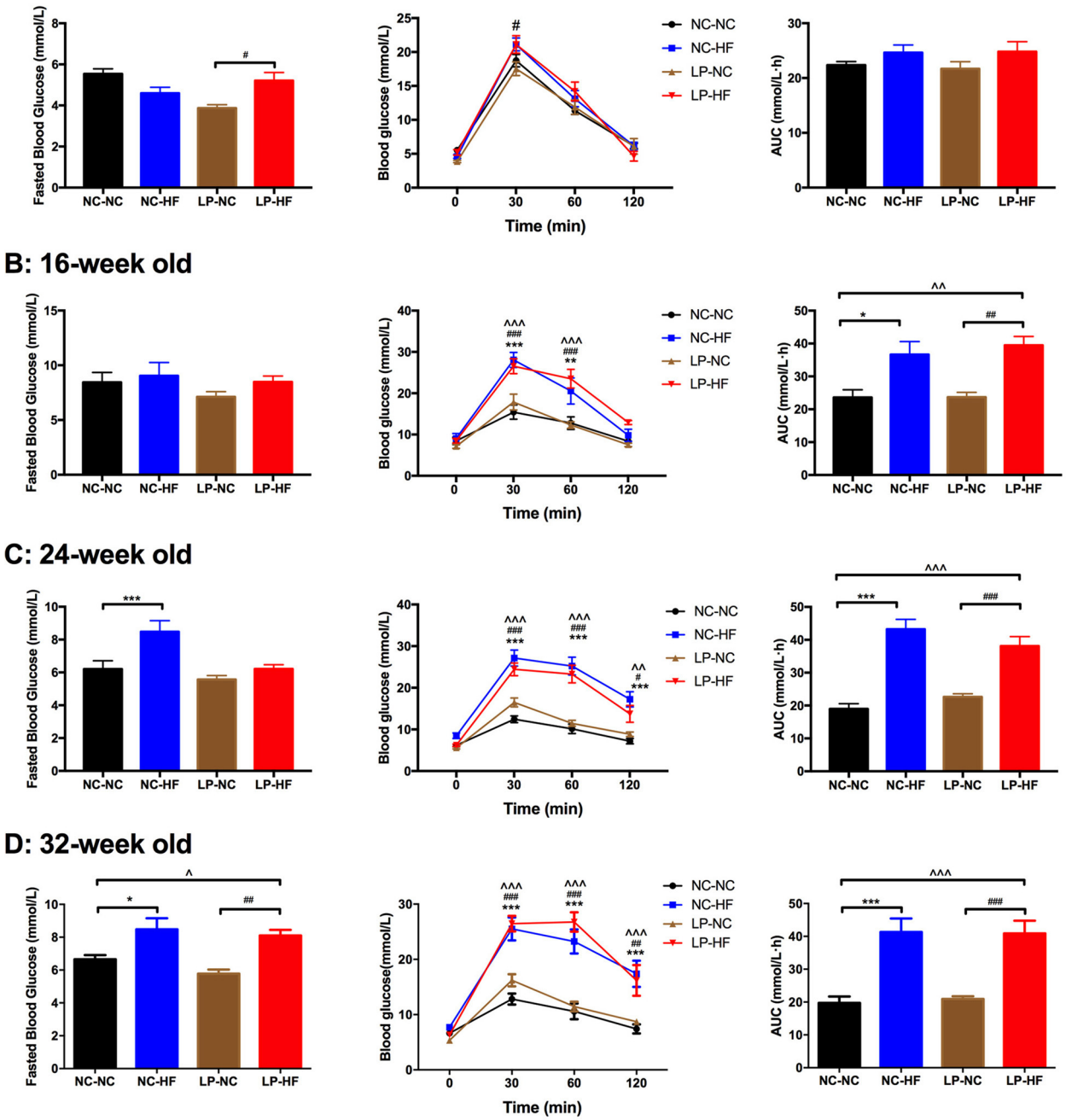

FIGURE 2 | Long-term effects of maternal protein restriction and post-weaning high-fat feeding on fasted blood glucose and glucose tolerance in offspring from 8 to 32 weeks of age. **(A)** Glucose metabolism at 8 weeks of age; **(B)** glucose metabolism at 16 weeks of age; **(C)** glucose metabolism at 24 weeks of age; **(D)** glucose metabolism at 32 weeks of age. AUC: area under the curve of ipGTT; ipGTT: intraperitoneal glucose tolerance test. Data were represented as mean ± SEM (n = 6–8/group). $^{*}P < 0.05$, $^{**}P < 0.01$ NC–HF *vs.* NC–NC group, $^{***}P < 0.001$ NC–HF *vs.* NC–NC group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ LP–HF *vs.* LP–NC group. $^{\wedge\wedge}P < 0.01$ LP–HF *vs.* NC–NC group, $^{\wedge\wedge\wedge}P < 0.001$ LP–HF *vs.* NC–NC group. Diet abbreviations: NC, normal chow; LP, low protein; HF, high fat. Dam and pup diets denoted before and after the dash line, respectively.

(%) and fasted blood glucose ($r = -0.03$, $P = 0.92$) (**Figure 6A**). Remarkably, it indicated that POMC-specific methylation (%) was negatively correlated with the glucose response to a glucose load in offspring mice ($r = -0.42$, $P = 0.039$) (**Figure 6B**).

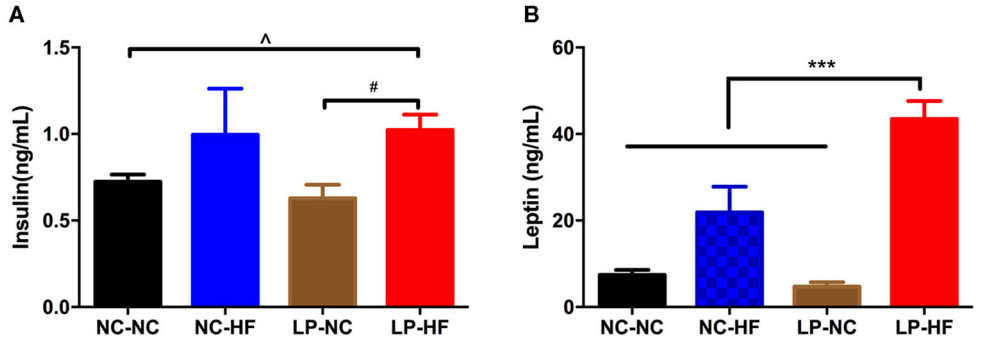

FIGURE 3 | Maternal protein restriction and post-weaning high-fat feeding predisposes offspring to hyperinsulinemia and hyperleptinemia. **(A)** Serum insulin level; **(B)** serum leptin level. Data were represented as mean ± SEM (n = 6–8/group). P-value is significant as denoted. Diet abbreviations: NC, normal chow; LP, low protein; HF, high fat. Dam and pup diets denoted before and after the dash line, respectively. $^{***}P < 0.001$ NC–HF *vs.* NC–NC group. $^{\#}P < 0.05$ LP–HF *vs.* LP–NC group. $^{\wedge}P < 0.05$ LP–HF *vs.* NC–NC group.

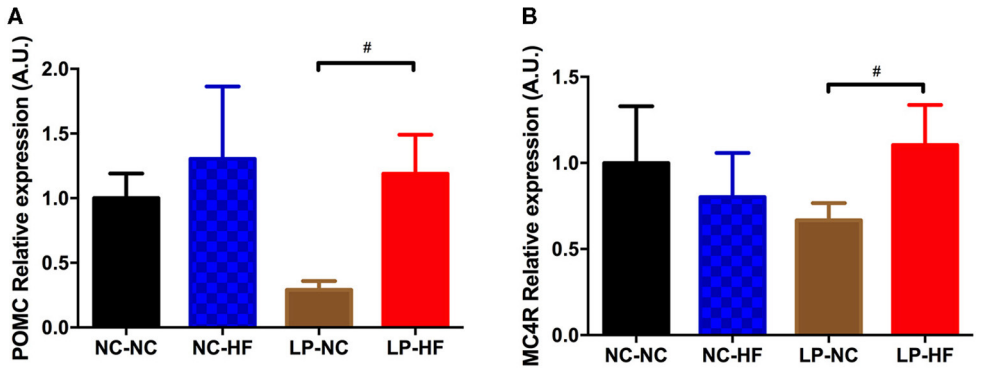

FIGURE 4 | Effects of maternal protein restriction and post-weaning high-fat feeding on hypothalamic gene expressions in offspring. **(A)** POMC; **(B)** MC4R. Data were represented as mean ± SEM (n = 6–8/group). $^{\#}P < 0.05$ LP–HF *vs.* LP–NC group. Diet abbreviations: NC, normal chow; LP, low protein; HF, high fat. Dam and pup diets denoted before and after the dash line, respectively.

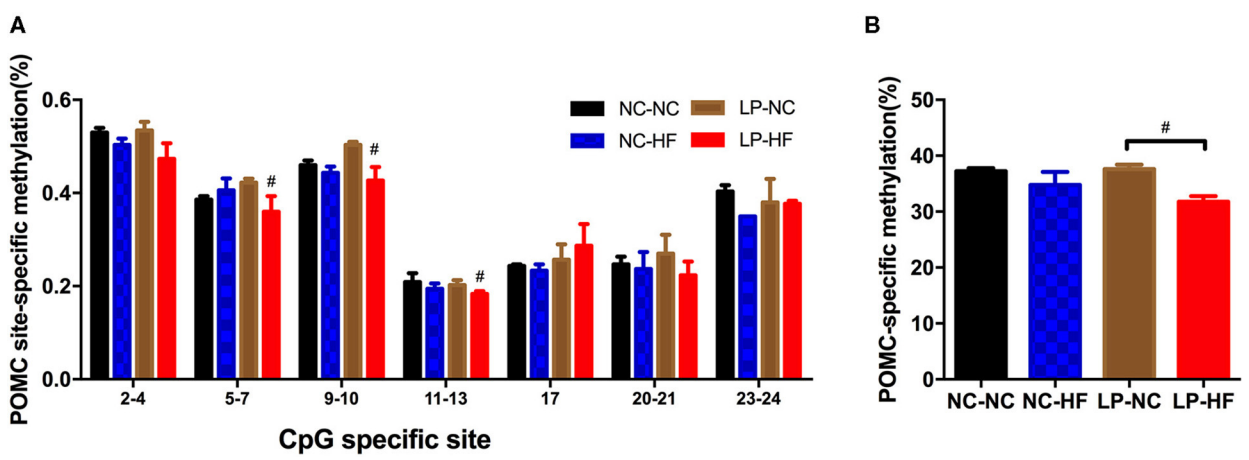

FIGURE 5 | Effects of maternal protein restriction and post-weaning high-fat feeding on POMC methylation in offspring. **(A)** Methylation level (%) of specific CpG site in POMC gene promoter; **(B)** POMC-specific methylation (%). POMC site-specific methylation (%). Data were represented as mean ± SEM (n = 6–8/group). $^{\#}P < 0.05$ LP–HF *vs.* LP–NC group. Diet abbreviations: NC, normal chow; LP, low protein; HF, high fat. Dam and pup diets denoted before and after the dash line, respectively.

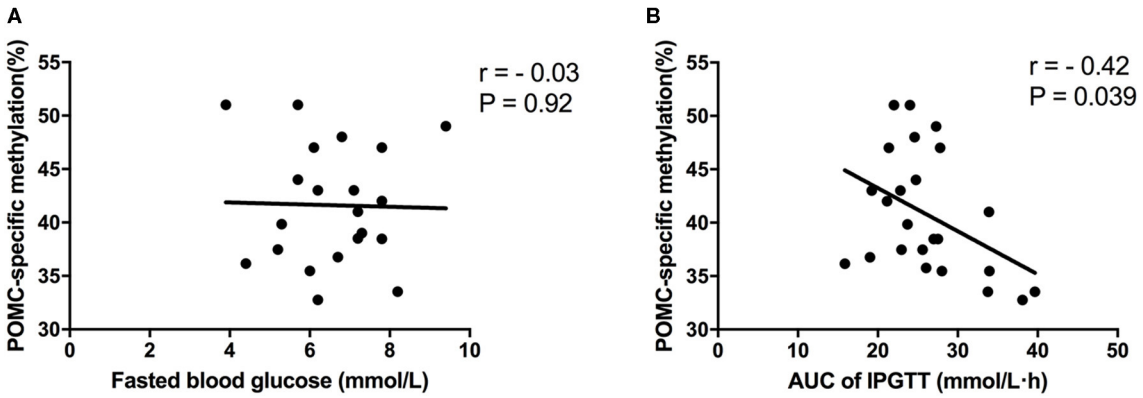

FIGURE 6 | Correlation analyses between POMC methylation and glucose metabolism status. **(A)** POMC-specific methylation (%) and fasted blood glucose; **(B)** POMC-specific methylation (%) and AUC of ipGTT. Data were represented as mean ± SEM (n = 6–8/group). Diet abbreviations: NC, normal chow; LP, low protein; HF, high fat. Dam and pup diets denoted before and after the dash line, respectively.

DISCUSSION

It has been widely reported that prenatal LP and postnatal HF diets can induce the occurrence of long-term metabolic disorders in mammals, including obesity, glucose intolerance, and type 2 diabetes (24, 25). In our study, we showed that maternal LP diet induced adaptive changes, leading to obesity, impaired glucose tolerance, hyperinsulinemia, and hyperleptinemia when the mice were challenged with post-weaning high calorie intake. Consistently, previous studies showed that maternal and post-weaning imbalanced nutrition induced detrimental consequences on glucose homeostasis in adult life (1, 2, 26, 27). However, there is no difference in glucose tolerance between maternal LP diet and NC diet, both together with a post-weaning HF. It seems that the impaired glucose homeostasis mainly results from the HF feeding. Thus, a post-weaning HF diet results in the development of aberrant energy homeostasis and metabolic diseases in later life.

The central nervous system, mainly hypothalamus is the central control of whole body energy homeostasis (28). The arcuate nucleus in hypothalamus contains both anorexigenic and orexigenic neurons, which can counterbalance each other to regulate food intake and energy expenditure, and ultimately control body weight (29). POMC neuron, as the most-studied anorexigenic neuron in the arcuate nucleus, it can regulate energy homeostasis *via* MC4R in the paraventricular nucleus (30). It indicates that the peptides released from POMC neuron clearly play a role in reducing food consumption to control body weight. Increasing evidence has indicated that exposure to adverse maternal nutrition impairs hypothalamic development and function, which is plastic and sensitive to metabolic signals, potentially underpinning metabolic health in adult life (31). Our study indicated that both hypothalamic POMC and MC4R expressions significantly increased in offspring mice that were exposed to maternal protein restriction combined with post-weaning HF feeding. In addition, we found that serum insulin and leptin levels were significantly increased in HF-fed offspring whose mothers had eaten the LP diet. Consistent with our findings, Ikenasio-Thorpe et al. showed that prenatal under-nutrition (30% of *ad libitum* intake throughout gestation) and postnatal HF nutrition (45% kcal as fat) in Wistar rats exhibited increased food intake, obesity, and higher fat mass in offspring at 24 weeks of age, which correlated with hypothalamic POMC increment and circulating insulin and leptin level elevations (32).

Furthermore, we investigate the epigenetic status of POMC and MC4R gene in offspring mice. It indicated that hypothalamic POMC promoter methylation was significantly decreased in mice exposed to maternal LP diet and post-weaning HF feeding. However, no CpG island was detected of MC4R gene promoter, thus it restricted to evaluate methylation status of MC4R gene. It is widely acknowledged that hypomethylation of a certain gene can activate transcription and increase gene expression (33). In our study, we found that decreased methylation of POMC gene promoter was consistently related with increased gene expression in hypothalamus. Consistently, Stevens et al. showed a marked hypomethylation (62% decrease) of hypothalamic POMC promoter in the ovine fetus exposed to maternal diet intake restriction (34). It is widely accepted that epigenetic modifications mainly occur during early life development, which may continue throughout the lifespan (35). Our study indicated that maternal malnutrition and post-weaning HF diet can regulate epigenetic modifications in offspring mice at 32 weeks of age. This provides evidence that DNA methylation could play as a programming mechanism for hypothalamic POMC gene, which can regulate abnormal glucose metabolism through hypothalamic feeding center in later life. Of interest, our present study showed that POMC-specific methylation (%) was negatively correlated with the competence of glucose response to a glucose load. It indicated that POMC promoter methylation may be a critical epigenetic modification which can project to regulate food intake, body weight, and glucose metabolism in the next generation.

In conclusion, our study indicated that maternal LP diet combined with post-weaning HF feeding resulted in obesity, persistent glucose intolerance, hyperinsulinemia, and hyperleptinemia in offspring. We further found that POMC gene methylation status may be a potential mechanism for impaired glucose metabolism in offspring. These findings can advance our thinking about hypothalamic POMC gene methylation between maternal LP diet combined with post-weaning HF feeding and metabolic health in offspring.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Peking University First Hospital Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JZha conceived and designed the experiments. JZhe, LZ, and JL carried out the experiments. JZhe and YL analyzed the data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.657848/full#supplementary-material

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Effects of Fermented *Radix puerariae* Residue on Nutrient Digestibility and Reproductive Performance of Sows

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This study was conducted to investigate the effect of fermented *Radix puerariae* residue (FRPR) on reproductive performance, apparent total tract digestibility (ATTD) of nutrients, and fecal short-chain fatty acid (SCFA) contents of sows. A total of 36 landrace × large white multiparous sows were randomly arranged into three treatments, representing supplementation with 0, 2, and 4% FRPR to a corn-soybean meal and wheat bran-based diet during the whole gestation period. The results showed that dietary FRPR had no effects on litter size and the number of total alive piglets ($P > 0.05$), and that the number of weaned piglets and weaning weight of litter were increased in sows with 4% FRPR treatment compared with control treatment ($P < 0.05$). Dietary 4% FRPR significantly decreased constipation rate, improved the ATTD of dry matter and organics, and fecal contents of acetate, propionate, and total SCFAs ($P < 0.05$). In the offspring piglets, serum concentrations of total protein, alkaline phosphatase, IgG, IL-10, and TGF-β were increased, but blood urea nitrogen content was decreased with 4% FRPR treatment ($P < 0.05$). There were no significant differences in all determined indexes except for fecal acetic acid and total SCFAs between control and 2% FRPR treatment ($P > 0.05$). These findings indicated that FRPR used in the diets of sows showed positive effects on fecal characteristics, utilization of nutrients, and reproductive performance. Maternal supplementation with 4% FRPR is recommended for improving immune responses, weaning litter size, and litter weight of offspring piglets, which provide useful information for the application of residues of *R. puerariae*.

Keywords: fermented *Radix puerariae* residue, nutrient digestibility, reproductive performance, sows, offspring

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INTRODUCTION

Pueraria lobata Ohwi (Latin name *Radix puerariae*), a Chinese traditional medicine, has always been used to treat diabetic nephropathy, cardiovascular diseases, Parkinson's disease, Alzheimer's disease, and acute cerebral ischemic stroke (1). Its roots contain many kinds of isoflavones, triterpenes, saponins, and so on. After the extraction of these substances, there are still lots of cellulose and some active components that remain in residues. The dregs of *R. puerariae* have been determined to contain 4.79% crude protein, 58.08% neutral detergent fiber, 22.96% acid detergent fiber, and 1.18% flavonoids. More than 18 tons/acre of *R. puerariae* are produced, but millions of tons of residues are discarded, which is a huge waste and can also lead to serious

pollution. Fermentation is an important way of boosting the comprehensive utilization of the residue of *R. puerariae* and other feeds (2). Fermented *Radix puerariae* residue (FRPR) may be an unconventional feedstuff for livestock, especially as a fiber source in sows, due to its possible effects on gut health and welfare.

Under commercial intensive feeding conditions, pregnant sows are often limit-fed to prevent excessive weight gain, which can lead to negative consequences on locomotion, farrowing, and post-farrowing feed intake (3, 4). However, this kind of restricted feeding strategy may induce aggression and stereotypies (5), or even affect the health of offspring piglets. There is evidence that fiber-rich diets could increase the satiety of pregnant sows and, thereby, reduce restricted feeding-induced behavioral problems (6, 7). Moreover, fiber-rich diets for sows during pregnancy could also reduce agonistic interactions among piglets (7). Except for the benefits of animal welfare, fiber-rich diets during gestation can also improve the reproductive performance of sows and the growth performance of piglets (8, 9). Moreover, a fiber-rich diet for gestating sows during transition reduced the proportion of stillborn piglets and mortality of total born piglets (10).

Studies have shown that the nutritional composition of colostrum can be changed with additives or fiber type and content in the diet of pregnant and lactating sows, thereby regulating the growth performance and immunity of offspring piglets (11–13). In view of that, this study aims to determine the effects of dietary FRPR on reproductive performance, apparent total tract digestibility (ATTD) of nutrients, and short-chain fatty acids (SCFAs) of sows. Serum biochemical parameters and cytokine concentrations of offspring piglets are also examined to evaluate the health status of piglets after maternal dietary supplementation of FRPR.

MATERIALS AND METHODS

Fermented *Radix puerariae* Residue Preparation

Dietary fermented *Radix puerariae* residue was prepared using residues from the roots of *Pueraria lobata* (Wild) *Ohwi* at the College of Animal Science, Jiangxi Agricultural University (Nanchang, Jiangxi, China). Residues from roots of *Radix puerariae* were autoclaved at 121°C for 1.5 h and inoculated with a liquid strain of *Trichoglossum* spp. after cooling. They were cultured at 25–27°C in a dark room for 25 days, and then dried and pulverized. The final FRPR contained 12.5% crude protein, 38.1% crude fiber, and 7.75% soluble fiber analyzed using Association of Official Agricultural Chemists (AOAC) methods.

Animals and Experimental Design

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Hunan, China. The animal protocol was approved by Institutional Animal Care and Use Committee (IACUC No. 20190056). Thirty-six 3–4 parity Landrace × Large White multiparous sows housed in individual stalls were randomly assigned into three treatments (control, 2% FRPR, and 4% FRPR) with 12 sows/treatment and fed with diet supplementation with 0, 2, and 4% FRPR, respectively. The diets were formulated to meet the nutrient requirements for the early and late stages of pregnancy for sows and contain equal nitrogen and energy (**Table 1**). Wheat bran of 0, 2, and 4% were replaced by FRPR, and the final crude fiber contents were 5, 5.55, and 6.15% (days 0–84 of gestation), and 3.5, 4.1, 4.7% (days 85–114 of gestation). The sows were fed with individual diet twice a day based on their body weight as follows: 1.7–2.2 kg/days from mating day to 30th day of gestation, 2.2–2.5 kg/days from 31st to 84th day of gestation, and 2.8–3.5 kg/days from 85th day of gestation to parturition day. On day 107 of gestation, all the sows were moved into an environmentally controlled farrowing room equipped with a feeder and a nipple drinker for sows, and as a nipple drinker and a heating lamp were provided for suckling piglets. All lactating sows were fed the same lactation diet and piglets were fed the same creep feed *ad libitum* until weaning at 21 days. All the sows and piglets had free access to water during the whole experimental period.

On day 77 of gestation, 0.3% of chromium trioxide was added to the diet as an indicator to determine nutrient digestibility. After a 3-day adaptation period, fecal samples were collected for 5 days. All fecal samples from the same sow were mixed, dried at 65°C, and pulverized for chemical composition detection, and fresh fecal samples of sows were collected by rectal massage on day 84 of gestation for the analysis of SCFA concentrations. Blood samples were collected from 21-day-old piglets from the jugular vein, and serum samples were obtained by centrifugation at 3,000 × g for 10 min at 4°C and then immediately stored at −80°C for further analysis.

Constipation Rate and Reproductive Performance of Sows

The judgment criteria for constipation in sows is dry, hard, and ball feces, which is manifested by loss of water, reduction in volume, change in texture, and darkening of color. Constipation rate in each treatment every day during the whole pregnancy period was calculated as follows: constipation rate (%) = 100 × number of constipated sows/total number of sows. Reproductive performance, which included litter size, alive litter size, number of weaning piglets, litter weight of birth, and weaning on day 21 age, was recorded and calculated.

Apparent Total Tract Digestibility of Nutrients

Feed and fecal samples were analyzed for dry matter, total energy, crude protein, neutral detergent fiber (NDF), acid detergent fiber (ADF), organics, and crude fat according to AOAC methods. The concentration of chromium trioxide in the diet and fecal samples was measured photometrically and used to calculate nutrient digestibility.

Abbreviations: FRPR, fermented Radix puerariae residue; SCFAs, short-chain fatty acids; NDF, neutral detergent fiber; ADF, acid detergent fiber; Ig, immunoglobulins; IL, interleukin; TNF-α, tumor necrosis factor alpha; TGF-β, transformed growth factor beta; IFN-γ, interferon-gamma.

TABLE 1 | Ingredient composition of diets in early and later stages of pregnancy.

Items	Day 0–84 of gestation			Day 85–114 of gestation		
	Control	2%FRPR	4%FRPR	Control	2%FRPR	4%FRPR
Ingredient, %						
Corn	52.80	53.00	52.80	63.80	63.50	63.20
Soybean meal (43% CP)	11.60	11.40	11.60	23.50	23.70	23.90
Wheat bran	30.00	28.00	26.00	4.00	2.00	-
Puerarin fiber	/	2.00	4.00	/	2.00	4.00
Lithopone powder	1.30	1.30	1.30	1.00	1.00	1.00
Calcium hydrogen phosphate	1.20	1.20	1.20	1.20	1.20	1.20
Sodium chloride	0.40	0.40	0.40	0.40	0.40	0.40
Sodium bicarbonate	0.20	0.20	0.20	0.20	0.20	0.20
Soybean oil	0.50	0.50	0.50	1.90	2.00	2.10
Premix*	2.00	2.00	2.00	2.00	2.00	2.00
Fish meal	/	/	/	2.00	2.00	2.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient**						
Digestible energy (DE, Mcal/kg)	2.93	2.93	2.93	3.34	3.35	3.34
Net energy (NE, Mcal/kg)	2.21	2.20	2.20	2.50	2.50	2.50
Crude protein (CP, %)	13.60	13.51	13.51	17.02	17.02	17.02
Crude fiber (CF, %)	5.00	5.55	6.15	3.50	4.10	4.70
Lystine (Lys, %)	0.70	0.70	0.70	1.08	1.09	1.09
Methionine (Met, %)	0.23	0.22	0.22	0.41	0.41	0.41
Tryptophan (Try, %)	0.17	0.16	0.16	0.24	0.24	0.24
Calcium (Ca, %)	0.88	0.89	0.88	0.87	0.87	0.88
Total phosphorus (TP, %)	0.66	0.66	0.66	0.58	0.58	0.57
Non-phytate phosphorus (NP, %)	0.35	0.35	0.35	0.38	0.38	0.38

**Premix was different between pre-pregnancy and late pregnancy. The premix for pre pregnancy provides 15 mg/kg Cu, 150 mg/kg Fe, 95 mg/kg Zn, 60 mg/kg Mn, 0.45 mg/kg Se, 11,000 IU/kg VA, 2,000 IU/kg VD_3, and 100 mg/kg VE. The premix for late pregnancy provides 15 mg/kg Cu, 125 mg/kg Fe, 90 mg/kg Zn, 60 mg/kg Mn, 0.45 mg/kg Se, 7,000 IU/kg VA, 2,000 IU/kg VD_3, and 170 mg/kg VE.*

***Values of nutrients are calculated.*

TABLE 2 | Effect of dietary supplementation with fermented *Radix puerariae* residue (FRPR) on reproductive performance of the sows[1].

Items	Treatment			SEM	*P*-valve
	Control	2% FRPR	4% FRPR		
Average constipation rate, %	40	20	0	/	/
Litter size	11.25	11.85	11.95	0.50	0.616
Alive litter size	10.40	11.30	11.80	0.41	0.088
Number of weaning piglets on day 21 age	9.80^{b}	10.85^{ab}	11.35^{a}	0.41	0.046
Litter weight of birth, kg	16.24	16.71	16.93	2.16	0.974
Litter weight of weaning piglets on day 21 age, kg	62.24^{b}	68.45^{ab}	70.95^{a}	2.38	0.045

[1]*Values are mean ± SEM; n = 12.* ^{a,b}*Values with different letters within the same row are significantly different (P < 0.05).*

Fecal SCFA Concentrations in Sows

Concentrations of fecal short-chain fatty acidss, such as acetic, propionic, butyric, isobutyric a, valeric, and isovaleric, were analyzed as described in the previous study (14).

Serum Biochemical Parameters and Cytokines Concentrations in Piglets

Serum immunoglobulins (IgG and IgM), as well as biochemical parameters (total protein, albumin, blood urea nitrogen, glucose, alanine transaminase, aspartate aminotransferase, alkaline phosphatase, triglyceride, and cholesterol) of the piglets were determined using a biochemical analytical instrument (Beckman CX4, Beckman Coulter Inc., Brea, CA, United States) and commercial kits (Sino-German Beijing Leadman Biotech Ltd., Beijing, China). Concentrations of interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and interferon-gamma (IFN-γ) in the serum of piglets were measured using

Porcine Cytokine Array Q1 (QAP-CYT-1) (RayBiotech, Inc., Guangzhou, China) according to the instructions of the manufacturer. The average value of piglets in the same litter was analyzed as a biological duplication.

Statistical Analysis

Data were analyzed by ANOVA using the SPSS 17.0 software (SPSS, Inc., Chicago, IL, United States). Tukey's test was performed for multiple comparisons. The results were expressed as mean ± SEM, and statistically significant differences were assumed with $P < 0.05$.

RESULTS

Effect of Dietary FRPR Supplementation on Reproductive Performance of Sows

As shown in **Table 2**, there are no constipated sows in 4% FRPR treatment, while the constipation rates of 2% FRPR treatment and control treatment are 20 and 40%, respectively. The number of weaning piglets and litter weight at the age of 21 days were increased in 4% FRPR treatment compared with control treatment ($P < 0.05$). Dietary supplementation with FRPR did not influence litter size, alive litter size, and litter weight at birth ($P > 0.05$).

Effects of Dietary FRPR on ATTD of Nutrients in Diet of Sows

The digestibility of most nutrients, such as total energy, crude protein, neutral detergent fiber, acid detergent fiber and crude fat, was not significantly different among all the three treatments ($P > 0.05$). Dietary 4% FRPR supplementation significantly increased the digestibility of dry matter and organics ($P < 0.05$), but no significant differences were found between the 2% FRPR and control treatments ($P > 0.05$) (**Table 3**).

Effects of Dietary FRPR Supplementation on Fecal SCFA Concentrations of Sows

The results for fecal SCFAs (acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate) concentrations are presented in **Table 4**. Compared with the control treatment, fecal acetic acid and total SCFA concentrations in the sows with the 2 and 4% FRPR treatments were significantly increased ($P < 0.05$). The dietary 4% FRPR supplementation also significantly increased fecal propionic acid concentration compared with control sows ($P < 0.05$).

Effects of Maternal Dietary FRPR Supplementation on Serum Biochemical Parameters in Offspring Piglets

As shown in **Table 5**, compared with the control treatment, the maternal dietary 4% fermented *Radix puerariae* residue supplementation significantly increased serum concentrations of total protein, alkaline phosphatase, and IgG but decreased blood urea nitrogen in the offspring piglets ($P < 0.05$). There were no significant differences in determined serum biochemical parameters between control and the 2% FRPR treatment ($P > 0.05$).

Effects of Maternal Dietary FRPR Supplementation on Serum Concentrations of Cytokines in Offspring Piglets

To evaluate the immune status of the offspring piglets, serum concentrations of some cytokines were examined. The results showed that the maternal dietary 4% FRPR supplementation significantly increased ($P < 0.05$) serum IL-10 and TGF-β concentrations of the offspring piglets, but did not affect ($P > 0.05$) other inflammatory cytokines compared with the control treatment. No significant differences in cytokines were found between the piglets of 2% FRPR and control treatments ($P > 0.05$) (**Figure 1**).

DISCUSSION

The reproductive performance of sows is highly associated with profits from pig husbandry. Even a slightest increase in the number of piglets weaned per sow per year has considerable commercial advantages (8). Many studies have highlighted the beneficial effects of a high fiber diet on the reproductive performance of sows (15–20). Dietary supplementation with a by-product from *R. puerariae* is expected to improve the

TABLE 3 | Effects of dietary supplementation with FRPR on nutrients digestibility of sows[1].

Items	Treatments			SEM	*P*-valve
	Control	2% FRPR	4% FRPR		
Dry matter, %	78.97^{b}	80.48^{ab}	83.97^{a}	1.37	0.047
Total energy, %	88.76	89.21	90.15	1.85	0.938
Crude protein, %	87.68	87.54	89.45	1.17	0.522
NDF, %	82.57	83.57	82.46	1.127	0.801
ADF, %	79.85	81.24	78.59	0.99	0.207
Organics, %	87.59^{b}	88.69^{ab}	91.89^{a}	1.20	0.046
Crude fat, %	56.57	56.78	57.67	0.71	0.561

[1]*Values are mean ± SEM; n = 12.* ^{a,b}*Values with different letters within the same row are significantly different (P < 0.05).*

TABLE 4 | Effects of dietary supplementation with FRPR on short-chain fatty acid (SCFA) contents in feces of the sows[1].

Items	Treatments			SEM	*P*-valve
	Control	2% FRPR	4% FRPR		
Acetic acid, mg/g	2.82[b]	4.87[a]	5.97[a]	0.54	0.001
Propionic acid, mg/g	1.79[b]	2.67[ab]	4.64[a]	0.55	0.006
Butyric acid, mg/g	1.38	1.78	1.97	0.39	0.606
Valeric acid, mg/g	0.44	0.67	0.75	0.09	0.052
Isobutyric acid, mg/g	0.29	0.38	0.39	0.06	0.468
Isovaleric acid, mg/g	0.67	0.69	0.78	0.09	0.670
Total SCFAs, mg/g	7.39[b]	11.06[a]	14.5[a]	1.58	0.013

[1]*Values are mean ± SEM; n = 12.* [a,b]*Values with different letters within the same row are significantly different (P < 0.05).*

TABLE 5 | Effects of maternal dietary supplementation with FRPR on serum biochemical parameters in the offspring piglets[1].

Items	Treatments			SEM	*P*-valve
	Control	2% FRPR	4% FRPR		
Total protein, g/L	46.17[b]	49.34[ab]	57.14[a]	2.80	0.031
Albumin, g/L	32.14	34.24	35.48	2.65	0.676
Blood urea nitrogen, mmol/L	5.67[a]	4.87[ab]	4.08[b]	0.42	0.046
Glucose, mmol/L	6.21	6.53	6.78	0.65	0.838
Alanine transaminase, U/L	34.27	37.12	39.59	5.34	0.798
Aspartate aminotransferase, U/L	29.34	30.59	34.38	2.37	0.325
Alkaline phosphatase, U/L	201.14[b]	235.47[ab]	275.61[a]	18.62	0.037
Triglyceride, mmol/L	0.75	0.69	0.72	0.08	0.856
Cholesterol, mmol/L	2.86	2.76	2.68	0.25	0.891
IgG, g/L	1.64[b]	1.76[ab]	2.05[a]	0.11	0.038
IgM, g/L	0.46	0.51	0.49	0.09	0.940

[1]*Values are mean ± SEM; n = 12.* [a,b]*Values with different letters within the same row are significantly different (P < 0.05).*

reproductive performance and shows beneficial effects on satiety and behavior as other fiber sources in sow (21).

Although the maternal dietary FRPR supplementation did not affect litter size and litter weight at birth, the 4% FRPR treatment significantly increased the number and litter weight of weaning piglets. These results were consistent with previous studies, such as the addition of 13.35% ground wheat straw to the gestation diet improving litter size and total litter weight at birth and weaning (5, 22). Dietary fiber has been demonstrated to improve oocyte quality and early embryo survival rate, increase litter size, prevent gestation and miscarriage, and improve the reproductive performance of sows (16–18, 20). High dietary fiber reduced estrogen during follicular development that could improve the survival rate of early embryos and reduce intrauterine growth retardation of fetal pigs, even stimulated the immune system, and reduced diarrhea rate and mortality (15).

Based on fecal characteristics, no sows in the 4% FRPR treatment had constipation, but the sows in the control treatment had a 40% constipation rate. Constipation may exaggerate the release and absorption of bacterial endotoxins and, thereby, the development of postpartum dysgalactia syndrome in sows (23). Another serious consequence of constipation is increased farrowing duration of sows due to the pain and discomfort caused by constipation (24). It has been suggested that farrowing duration was directly associated with the number of stillborn (25). Sows with longer farrowing duration are at a greater risk for having fever on the first day after parturition (26). It has been reported that a high-fiber diet increases fecal water content (27), which is a mutual confirmation with the results.

Fully understanding the effects of dietary fiber on nutrient digestibility is essential for the proper usage of fiber-rich diets in gestating sows. Dietary fibers generally have a negative impact on nutrient and energy digestibility. Previous studies have revealed that sows fed with a high-fiber diet exhibited lower digestibility of dry matter, crude protein, gross energy, non-fibrous carbohydrates, and organic matter (10, 28). However, there are also some opposite results due to different dietary fiber sources and compositions, as well as physiological stages of animals. Research reported that dietary energy digestibility improved with an increase in dietary soluble fiber (29). Agyekum et al. also found that supplementing processed straw, which is rich in fiber, during late gestation improved the apparent total tract digestibility of dry matter and energy (5). The authors speculated that the processing of straw might have improved

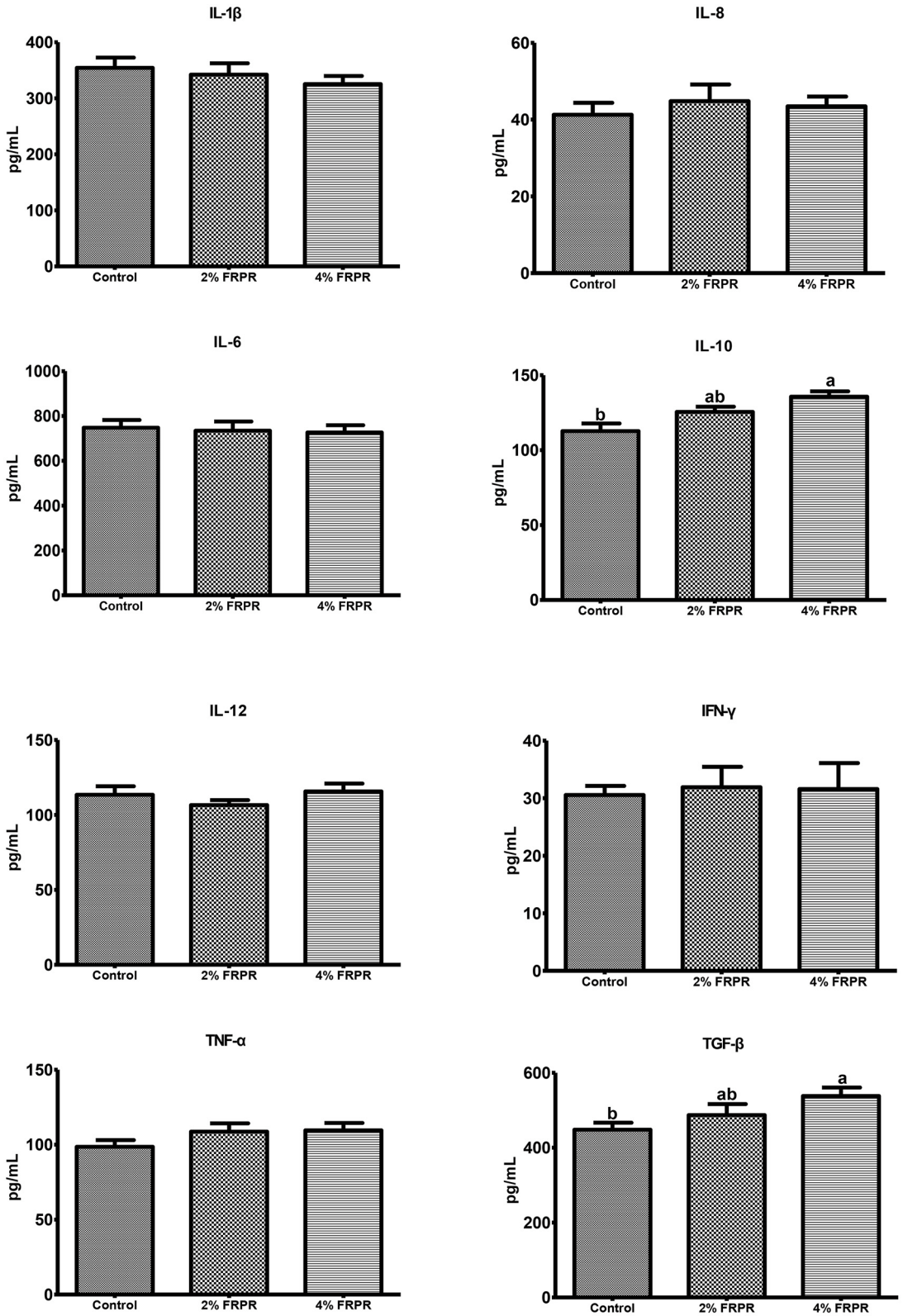

FIGURE 1 | Effect of maternal dietary supplementation with FRPR on serum concentrations of cytokines in offspring piglets. Values were means ± SEM, $n = 12$. [a,b]Values with different lowercase letters are different ($P < 0.05$).

digestibility by changing the fiber matrix, reducing fiber length, and opening up the cell wall structure. In this study, the dietary 4% FRPR supplementation significantly increased the digestibility of dry matter and organics. The relatively high soluble contents (7.75%) in the FRPR and fungal fermentation process may play important roles.

Dietary fiber is generally not hydrolyzed by endogenous enzymes but fermented by microbes in the hindgut (30). Major products of dietary fiber fermentation are SCFAs (acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, and isovaleric acid), which carry out diverse functional roles such as activating G-coupled-receptors, inhibiting histone deacetylases, and serving as energy substrates (31). Many studies have indicated that dietary fibers effectively alter intestinal microbiota composition and diversity in pregnant sows (9, 11, 21). Although in this study we failed to determine the intestinal microbial composition of sows, the results for fecal SCFAs may partially explain the regulation of dietary fiber on microbiota. In this study, the dietary 4% FRPR supplementation significantly increased fecal concentrations of acetic acid, propionic acid, and total SCFAs, which is consistent with the previous study that demonstrated that a high-fiber diet could increase fecal SCFA content (27, 32). Furthermore, the study showed that increasing dietary konjac flour, which is highly fermented by gut microbiota to produce SCFAs, linearly increased plasma SCFA concentration 4 h post prandial (33). Gut microbiota-derived SCFAs not only fuel host cells but also serve as signaling molecules between gut microbiota and extra-intestinal organs (34, 35). Notably, the recent findings showed that the gut microbiota of pregnant mice influence the immune and brain functions of offspring, raising the possibility that maternal SCFAs play a key role in the regulation of disease susceptibility during postnatal life (36, 37).

To investigate the maternal effects of dietary FRPR, serum biochemical parameters and cytokines concentrations in 21-day-old piglets were determined. The results showed that the maternal dietary FRPR supplementation influenced the metabolism and immune function of the offspring. The increase in serum total protein and decrease in blood urea nitrogen concentration indicated, to some extent, that the maternal FRPR supplementation improved protein synthesis and suppressed protein catabolism (38, 39), which were highly correlated with an increase in weaning weight of piglets. The increase in serum IgG concentration indicated that the immune status of piglets was improved by the maternal FRPR supplementation, which was further confirmed by a higher concentration of anti-inflammatory cytokine IL-10 and immune tolerance mediator TGF-β (40). Serum alkaline phosphatase activity was also increased in piglets of the FRPR treatment, verifying partially the probability for increase in the growth performance of piglets (41, 42).

Studies have identified that the isoflavone genistein could reduce intestinal cell proliferation *in vitro* and *in vivo* in piglets without affecting intestinal enzyme activity or nutrient transport (43). The contents of isoflavone in different sources of legumes feeds are significantly different, among different raw materials, which may reduce the severity of rhinovirus (RV) infections (44). Whether the effects of FRPR on sows and offspring piglets are due to isoflavones or other specific substances need further research.

CONCLUSIONS

In conclusion, FRPR is a suitable fiber source for gestating sows to improve reproductive performance, ATTD of nutrients, and gut SCFAs production. Maternal supplementation with 4% FRPR is recommended for improving immune responses, weaning litter size, and litter weight of offspring piglets, which provide useful information for the application of residues of *Radix Pueraria* in sows.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Hunan Agricultural University.

AUTHOR CONTRIBUTIONS

ZL: investigation, visualization, and writing of original draft. YZ: writing the review. XL: resources. LZ and QZ: conceptualization and methodology. JH, BT, and JW: supervision and editing. All authors contributed to the article and approved the submitted version.

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The Impact of Maternal High-Fat Diet on Bone Microarchitecture in Offspring

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The incidence of obesity in women of reproductive age has significantly increased over the past 100 years. There is a well-established connection between maternal obesity during pregnancy and an increased risk of developing non-communicable cardiometabolic diseases in her offspring. This mini-review focuses on evidence examining the effect of maternal high-fat diet (HFD) on skeletal development and bone health in later life in offspring. The majority of rodent studies indicate that maternal HFD generally negatively affects both embryonic bone development and bone volume in adult animals. Details surrounding the mechanisms of action that drive changes in the skeleton in offspring remain unclear, although numerous studies suggest that some effects are sex-specific. Human studies in this area are limited but also suggest that HFD during pregnancy may impair bone formation and increase fracture risk during childhood. Given the consequences of low bone mass and deranged bone microarchitecture for offspring, advances in our understanding of the developmental origins of bone health is critical in the battle against osteoporosis.

Keywords: early life nutrition, maternal obesity, developmental origins of health and disease (DOHaD), osteoporosis, osteoblast, osteoclast, bone marrow adipocytes, skeletal development

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INTRODUCTION

The prevalence of obesity over the past 100 years has dramatically increased, with obesity identified as the most common metabolic disorder. Globally, an estimated 600 million adults were obese (body mass index $\geq$ 30 kg/m^2), and 1.9 billion adults were overweight in 2015 (body mass index 25–30 kg/m^2) (1). The prevalence of obesity is expected to reach 1.12 billion individuals by 2030 (2). Obese individuals have an increased risk of morbidity from type 2 diabetes mellitus (T2DM), cardiovascular disease, specific cancers, and osteoarthritis (3).

The incidence of obesity in women of reproductive age has also increased. Maternal obesity is a significant risk factor for maternal, fetal, and neonatal morbidities, including miscarriage, preterm delivery, hypertension, pre-eclampsia, and gestational diabetes (4–6). Research in the field of developmental origins of health and disease (DOHaD) has highlighted that maternal obesity during pregnancy predisposes offspring to develop obesity and other non-communicable diseases, including T2DM, hypertension, and cardiovascular disease, later in adulthood (7). Paternal obesity also increases the risk of developing non-communicable diseases in offspring (8–10), and both maternal and paternal obesity have transgenerational effects on subsequent generations via epigenetic effects on the germline (10–12). Thus, obesity and its related comorbidities represent an increasing burden on healthcare systems.

Although not fully understood, the effect of maternal obesity on the development of various organs and tissues such as the brain, liver, kidney, endocrine pancreas, and skeletal muscle and their structure and function have been well-researched with the aid of animal models. In these models, maternal obesity is induced via a high-fat diet (HFD) (13, 14). Recently the effects of maternal HFD on bone mass and strength in offspring and the risk of developing osteoporosis later in life have been researched. This mini-review will discuss evidence that maternal HFD-induced obesity affects bone development and microarchitecture, focusing on recent advancements using rodent models, and will discuss the potential mechanisms involved.

EARLY BONE DEVELOPMENT AND IMPACT LATER IN LIFE

The skeleton develops *in utero* from mesenchymal condensations. Most of the skeleton forms via a process known as endochondral ossification: initially, a cartilaginous template forms, which is later progressively replaced by mineralized bone matrix. Cartilaginous growth plates continue to control the longitudinal growth of bones throughout neonatal and childhood growth, while the overall bone shape, mineralization, and microarchitecture are determined by the balance of bone formation by osteoblasts and bone resorption by osteoclasts in different locations. Longitudinal skeletal growth continues until the late teens in humans, ending when the growth plates fuse. However, bone mineral density (BMD) continues to increase slowly until peak bone mass is reached in the mid-20s to early-30s. Rodents continue to grow slowly for a longer portion of their life, but mice reach peak bone mass at ∼12-weeks of age. Bone mass begins to decline as bone resorption outpaces bone formation as we age. In women, there is a dramatic period of bone loss following menopause when reductions in sex hormone levels affect homeostasis. Skeletal size, BMD, and bone microarchitecture are largely determined by genetics, with up to 85% of the variation in BMD explained by genetic factors (15). However, various non-genetic factors also influence both bone accrual during growth and bone loss in later life. Nutrition is a major factor influencing both growth and bone mass and can have effects at all life stages. Exercise, or the effect of loading on the skeleton, plays a major role in bone accrual and retention.

Osteoporosis is characterized by low BMD and a high risk of fracture, and affects one-half of elderly women and about one-fifth of elderly men. While osteoporosis is considered a disease of aging, early life events and a failure to achieve maximal peak bone mass determined by an individual's genetics can significantly impact future osteoporosis risk. One line of evidence for long-term impacts of events earlier in life comes from studies in athletes. Baseball pitchers who develop larger, stronger bones in their throwing arm during early adulthood can retain better bone structure in this arm for 50 years following retirement from the game (16). Various drugs are available that effectively reduce fracture risk in people with osteoporosis; however, the majority are antiresorptive therapies that prevent further bone loss but do not enable the replacement of bone already lost (17). Anabolic treatments restore bone microarchitecture to some degree, but are expensive biologics so not available to everyone. Understanding risk factors for low bone mass and identifying people at high risk of fracture are important for preventing fracture-related morbidity and mortality in aging populations.

EFFECT OF MATERNAL HFD ON BONE IN RODENTS

Thirteen rodent studies considered the effects of maternal HFD on offspring bone development (**Table 1**). The majority of studies implemented the maternal HFD regime before mating (4–15 weeks before conception) and continued through pregnancy and lactation. However, in three studies, the maternal HFD-feeding window was exclusively during pregnancy and lactation and exclusively during lactation in one study.

Fetal and Neonatal Offspring

Offspring of dams fed a maternal HFD before and during pregnancy have evidence of skeletal developmental delay in late-gestation with decreased bone formation, bone volume, and BMD (18–21). Chen et al. have demonstrated that maternal HFD promotes cellular senescence in fetal calvarial osteoblasts cells, potentially suppressing fetal bone formation (18–20).

In both newborns and weanlings, exposure to maternal HFD resulted in increased total bone mass, BMD, and trabecular bone volume in long bones (22, 23). This phenotype is likely the result of increased osteoblast activity, as bone modeling is most active over this period of rapid growth (31). Increased bone mass in weanlings may be an indirect effect of maternal HFD, as these offspring consume more milk, and the milk consumed has a higher energy content compared to dams fed control diet (CD) (22). Additionally, Miotto et al. found higher concentrations of monounsaturated fatty acids in the long bones of offspring exposed to maternal HFD, which is likely to reflect the diet consumed by the dams; these lipid stores may have supported rapid bone growth (23).

Adult Offspring

From early adulthood, a pattern of sustained bone loss in offspring of dams fed HFD is reported. In most studies, offspring exposed to maternal HFD have reduced BMD and bone volume in long bones and vertebrae from as early as 8-weeks of age, which persisted over their lifetime (18, 21–23, 25, 30). Hafner et al. found that maternal HFD during lactation alone was sufficient to increase bone marrow adiposity (28). Maternal HFD decreased trabecular bone parameters in offspring (18, 21–23, 25, 30). However, this effect was not observed in all studies; two studies found increased femoral bone trabecular volume and increased cortical thickness following maternal HFD (24, 27). Notably, these two studies analyzed offspring who were young adults, as opposed to the studies that found decreased bone volume in mice who were considerably older.

Several studies demonstrated sex-specific variation in the effect of maternal HFD, with males more likely to exhibit a bone phenotype than females (18, 22, 24). Only one study exclusively found changes in bones in females following maternal HFD (25).

TABLE 1 | Studies investigating the effect of maternal HFD on offspring bone properties.

Dietary details[a]	Animal strain	Dietary intervention period	Offspring age	Main findings in maternal HFD vs. CD	Proposed mechanism(s) of action of maternal HFD on offspring bone properties	References
Fetal						
HFD (45% fat) CD (17% fat)	C57BL/6J mice	8 weeks before mating and pregnancy	E17.5	Decreased total bone volume and bone mineralisation, increased senescence markers, pro-inflammatory cytokines, and chemokines in calvarial osteoblasts.	Maternal HFD promotes osteo-progenitor senescence and expression of pro-inflammatory factors, which could impair fetal skeletal development.	(18)
HFD (45% fat) CD (17% fat)	Sprague-Dawley rats	10 weeks before mating and pregnancy	E18.5	Decreased bone formation and ossification in calvaria and vertebrae, and decreased potential for calvarial osteoblast differentiation.	Demonstrate decreased osteogenic differentiation via hypermethylation and decreased expression of HoxA10.	(19)
HFD (42% fat) CD (17% fat)	Sprague-Dawley rats	12 weeks before mating and pregnancy	E18.5	Increased expression of p53/p21-mediated cell senescence signaling-related genes and proteins in calvarial osteoblasts.	Hypothesis: increased cell senescence may result in decreased glucose metabolism and cell differentiation.	(20)
HFD (60% fat) CD (18% fat)	C57BL/6J mice	4 weeks before mating and pregnancy	E19	Decreased body length, total bone volume, long bone lengths, and BMD. Some effects are ameliorated by maternal antioxidant supplementation.	Hypothesis: increased oxidative stress leads to placental vascular damage and impaired osteogenic fetal signaling pathways.	(21)
Postnatal						
HFD (60% fat) CD (10% fat)	Sprague-Dawley rats	Pregnancy and lactation only	P1 and P21	Increased Tb.BV/TV at P1 and P21.	Hypothesis: increased bone volume is driven by increased osteoblast activity.	(22)
			5 and 15 weeks	Decreased femur length, Tb.BV/TV, at 15 weeks (males only). Increased osteoclast number and surface, and osteoclastogenesis *ex vivo*.	Demonstrate increased osteoclast activity in 15-week males.	(22)
HFD (41% fat) CD (17 % fat)	Wistar rats	10 weeks before mating, pregnancy, and lactation	P28	Increased BMD and fatty acid content in the femur at P28.	None.	(23)
			12 weeks	BMD, femoral bone strength and fatty acid content not different.	None.	(23)
HFD (45% fat) CD (18% fat)	C57BL/6J mice	6 weeks before mating, pregnancy, and lactation	14 and 26 weeks	Increased femoral Tb.BV/TV at 14 weeks, not different at 26 weeks. No difference in bone strength. MAR increased in males at 14 weeks.	May be sexually dimorphic mechanisms involved. Males had higher MAR and lower osteoclast activity at 14 weeks.	(24)
HFD (60%) CD (10%)	C57BL/6J mice	11–15 weeks before mating and during pregnancy and lactation	28 weeks (F1 and F2 offspring)	Decreased Tb.BV/TV and BMD in tibia in F1 and F2 female offspring. No changes in males.	None.	(25)
HFD (60% fat) CD (18% fat)	C57BL/6J mice	4 weeks before mating and during pregnancy and lactation	26 and 52 weeks (females only)	Decreased femoral BMD at 26 weeks, increased Tb.Sp at 52 weeks.	None.	(26)
Post-weaning crossover diet studies						
HFD (45% fat) CD (7% fat)	C57BL/6J mice	Pregnancy and lactation. Four groups at weaning: CD/CD, CD/HFD, HFD/CD, HFD/HFD	6 weeks	Increased femoral length, bone volume, and cortical thickness (males only); changes were amplified HFD/HFD. MGP expression negatively correlated with bone volume.	None.	(27)
HFD (45% fat) CD (17 % fat)	C57BL/6J mice	8 weeks before mating and during pregnancy and lactation. Four groups at weaning (as above)	17 weeks	Decreased Tb.BV/TV in all male HFD groups, increased CSA and medullary area in HFD/CD males.	Increased expression of senescence-related proteins in both ages. Early effects after maternal HFD persist into adulthood.	(18)

(Continued)

TABLE 1 | Continued

Dietary details[a]	Animal strain	Dietary intervention period	Offspring age	Main findings in maternal HFD vs. CD	Proposed mechanism(s) of action of maternal HFD on offspring bone properties	References
HFD (60% fat) CD (14% fat)	C57BL/6J mice	Lactation only. Weaned onto CD, 4 groups at 12 weeks (as above)	24 weeks	Decreased Tb.BV/TV in HFD/HFD only (males, females not analyzed). Lactational HFD increased bone marrow adiposity, further amplified in HFD/HFD.	Hypothesis: BMSCs are more committed to a pro-adipogenic lineage, resulting in greater bone marrow adiposity and decreased bone mass.	(28)
HFD (43% fat) CD (14% fat)	C57BL/6J mice	7 weeks before mating and during pregnancy and lactation. Three groups at weaning (no CD/HFD)	30 weeks	Femurs shorter in HFD/HFD, no difference in Tb.BV/TV. Increased number of bone marrow adipocytes and diameter of adipocytes (females only) in HFD/HFD vs. CD/CD.	None.	(29)
HFD (45% fat) CD (7% fat)	C57BL/6J mice	Pregnancy and lactation. Four groups at weaning (as above)	30 weeks	Femoral Tb.BV/TV decreased in HFD/HFD males. Vertebral Tb.BV/TV decreased in HFD/CD males. No changes in females.	None.	(30)

[a] *All shown as % kcal from fat.*

BMSC, bone marrow stromal cell; BMD, bone mineral density; CD, control diet; CSA, cross section area; E, embryonic day; F1/2, first/second generation offspring; HFD, high-fat diet; HoxA10, homeodomain-containing factor A10; MAR, mineral apposition rate; MGP, matrix gla protein; P, postnatal day; Tb.BV/TV, trabecular bone volume fraction; Tb.Sp, trabecular separation.

Therefore, maternal HFD most likely has a sexually dimorphic effect on the skeleton of offspring.

Multigenerational Effects of Maternal HFD

Maternal HFD can have multigenerational effects on bone in offspring and grand-offspring. Harasymowicz et al. found decreased trabecular bone volume and BMD in F1 and F2 generations, even with no additional exposure to HFD in either generation (25).

Postnatal Exposure to HFD

A post-weaning HFD, or a "second hit," has the potential to amplify the effects of maternal HFD (32). Three studies that investigated continued feeding of HFD in offspring after weaning found that post-weaning HFD further decreased trabecular bone volume (28, 30) or increased bone-marrow adiposity (28, 29), compared to exposure to maternal HFD alone.

EFFECT OF MATERNAL OBESITY AND HFD ON BONE IN HUMANS

Several studies have specifically addressed whether maternal obesity or maternal HFD during pregnancy affects bone development in offspring, both *in utero* and post-partum (33). Longitudinal studies show that obese mothers have babies with increased body length, whole-body bone area, and mineral content (34–36), but maternal diet was not reported. Two studies demonstrated that mothers consuming a high-fat "Western diet" during pregnancy, defined as a diet high in meat, processed food, and saturated fat, have children with lower whole-body bone area, bone mineral content, and BMD, compared with children of mothers on low-fat "prudent diets" during pregnancy, defined as a diet high in fruits, vegetables, grains and low-fat dairy products (37, 38). Interestingly, offspring of mothers in the Danish National Birth Cohort who consumed a Western diet had a significantly increased risk of fracture between birth and 16-years of age (39). None of these studies reported maternal BMI related to study groups. Due to the paucity of human data (33), it is unclear whether maternal obesity in the absence of HFD, maternal HFD in the absence of obesity, or any other dietary conditions of over-nutrition with or without maternal obesity affects the skeletal phenotype in human offspring.

MECHANISMS OF ACTION

Research into the mechanisms involved in linking maternal HFD with a bone phenotype in rodents remains in its infancy, and to our knowledge, no studies in humans have explored any mechanisms of action. The following section discusses some key mechanisms demonstrated in rodent maternal HFD studies, linking the early life environment and the observed bone phenotype in these offspring (**Figure 1**).

Osteoblasts

Osteoblasts are derived from mesenchymal stem cells and are responsible for the synthesis and mineralisation of bone. Whilst osteoblast number is unaffected (22, 24), there may be a negative relationship between osteoblast function in offspring and maternal HFD during pregnancy and lactation. However, this relationship with osteoblast function may be transient and lost as offspring age. Whole-embryo skeletal ossification and total bone volume are decreased following maternal HFD (18, 19). Rat calvarial osteoblasts from offspring exposed to maternal HFD have decreased proliferation and osteoblastic differentiation (19). Therefore, decreased differentiation of osteoblasts could

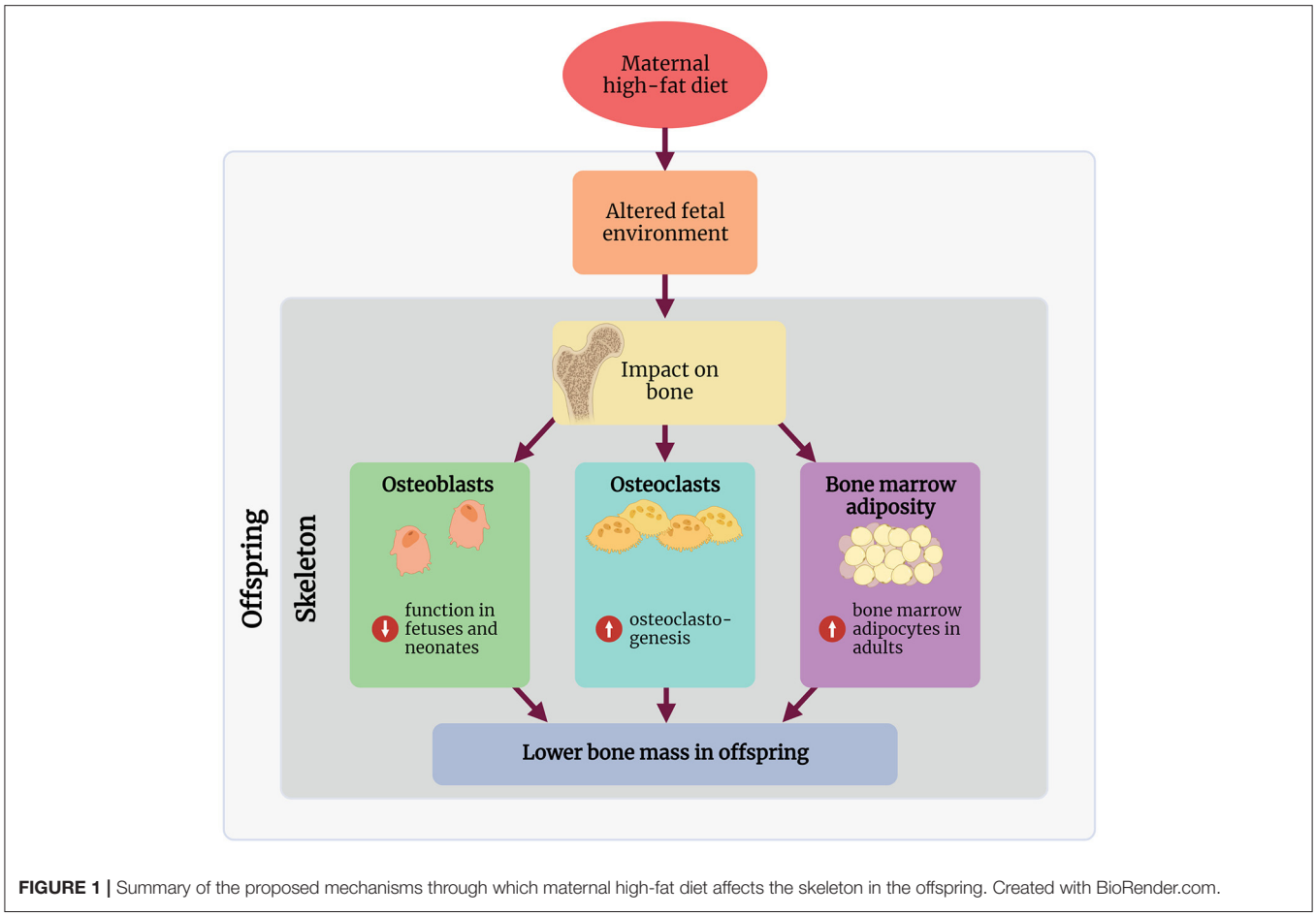

FIGURE 1 | Summary of the proposed mechanisms through which maternal high-fat diet affects the skeleton in the offspring. Created with BioRender.com.

directly be responsible for decreased or delayed bone formation during development.

Although bone marrow stromal cells (BMSCs) are a significant source of osteoblast progenitor cells contributing to bone remodeling, only one study examined whether the differentiation capacity of these cells into osteoblasts is influenced by maternal HFD. Kushwaha et al. assessed cellular activity in 15-week old animals and found no difference in osteogenic differentiation of BMSCs exposed to maternal HFD. However, compared to BMSCs derived from CD-fed mothers, these cells have higher mRNA expression of RANKL, which will have implications for osteoclastogenesis (22).

Osteoblast function in adult rodents exposed to maternal HFD is variable. Circulating osteocalcin concentrations are decreased at 17-weeks of age in mice, indicating osteoblast function is decreased following maternal HFD. Mineral apposition rate (MAR) is a reliable direct measurement of osteoblast function (40); one rat study found no difference in MAR in 15-week old males following maternal HFD (22). Interestingly, Devlin et al. found that MAR is increased in 14-week old male mice exposed to maternal HFD (24). This relationship is no longer detected in male mice at 26-weeks of age (24), indicating the rate of mineralization has decreased to a level similar to maternal CD offspring. Surprisingly, Devlin et al. also found no difference in concentrations of the bone formation marker type 1 procollagen N-terminal (P1NP) in circulation at either 14- or 26-weeks of age, despite observed differences in MAR (24). Overall, there is no consensus on whether maternal HFD affects osteoblast function in adult offspring.

Osteoclasts

Osteoclasts are multinucleated phagocytic cells responsible for bone resorption and are derived from the macrophage-monocyte cell lineage. Very few studies have considered the effects of maternal HFD on osteoclasts. One study broadly examined *ex vivo* osteoclastogenesis following maternal HFD during pregnancy and lactation (22). Kushwaha et al. demonstrated via histomorphometry that osteoclast number, erosion surface, and osteoclast surface were increased in 15-week old male rats exposed to maternal HFD. *Ex vivo* cultures of osteoclast precursors isolated from these animals had increased potential to differentiate into osteoclasts, with these osteoclasts more numerous and larger. Interestingly, these osteoclasts were more sensitized to the effects of RANKL and had increased RANK mRNA expression. Cultured osteoblasts from these same animals had increased RANKL mRNA expression, indicating that the

potential for osteoclastogenesis is increased following maternal HFD. Alternatively, when mice were fed HFD for 6-weeks before mating, pregnancy, and lactation, their offspring demonstrated no significant difference in osteoclast number but decreased osteoclast activity at 14- and 26-weeks of age (24). Increased bone resorption is a major contributor to decreased bone volume that develops when rodents are fed HFD, which is likely secondary to increased inflammation (41). Maternal HFD is known to cause low-grade chronic inflammation in offspring; therefore, it is feasible that this could contribute to bone loss in these animals (42). Chen et al. noted increased inflammatory cytokine production in fetal calvarial osteoblasts exposed to maternal HFD, but this potential mechanism has not been addressed in adult offspring (18). Given these conflicting data, further studies are needed to determine the effects of maternal HFD on osteoclast number and function.

Bone Marrow Adiposity

The balance between BMSCs giving rise to osteogenic or adipogenic precursors is critical for maintaining bone mass; if this balance is shifted toward adipogenesis, this may come at the expense of osteoblastogenesis (43). Additionally, increased bone-marrow adiposity can affect osteogenesis, with an apparent negative relationship between bone marrow adiposity and bone mass (44). Maternal HFD during pregnancy and lactation (29) or lactation only (28) is associated with increased adipocyte number and adipocyte size in the bone marrow cavity. However, interpretation of these studies is complicated by their study design; offspring were either weaned directly onto HFD or CD, or onto CD followed by HFD between 12 and 24-weeks of age. Both studies had conflicting results as to whether maternal HFD with post-weaning HFD affected bone microarchitecture. Hafner et al. found bone marrow adiposity was increased, and trabecular bone volume was decreased in the maternal HFD/post-weaning HFD group at 24-weeks of age (28). However, Lanham et al. found no difference in bone microarchitecture at 30-weeks of age (29). No studies performed *ex vivo* adipogenesis assays on BMSCs. Further studies are required to confirm whether changes in bone marrow adiposity contribute to the bone phenotype in these offspring.

Epigenetic Modifications

Epigenetic modifications, including DNA methylation and various post-translational histone modifications, describe changes to gene expression that occur without affecting the underlying DNA sequence. Epigenetic modifications allow the individual to alter gene expression in response to the environment and have long been considered a principal mechanism through which the early-life environment affects offspring (7, 45). Despite this connection, there is a paucity of studies that have specifically measured epigenetic modifications in response to maternal HFD.

Chen et al. demonstrated that maternal HFD promotes cellular senescence in fetal calvarial osteoblasts, potentially suppressing bone formation in the prenatal period in both mice and rats (18–20). These findings were shown to be through increased expression of p300/CBP, which increased H3K27 acetylation, which promoted p53/p21-mediated cell senescence signaling in pre-osteoblasts; increased expression of p300/CPB persisted until adulthood (18). Maternal HFD also promoted increases in methylated CpG sites in the homeobox protein A10 (HoxA10) promoter. HoxA10 is important for fetal osteoblastogenesis and adult bone regeneration.

CURRENT RESEARCH GAPS

Many unanswered questions surrounding how HFD-induced maternal obesity affects bone development and microarchitecture in offspring remain.

One outstanding question is whether the detrimental effects on offspring skeleton are driven by maternal obesity, maternal HFD, or both. In humans, most studies explore the effects of obesity during pregnancy, commonly assessed by measuring body mass index rather than dietary patterns (46). In rodents, the majority of studies implemented a maternal HFD regime at least 4 weeks before mating. This would have induced an obesity phenotype in these dams, as well as ongoing exposure to HFD. However, it is unlikely that maternal obesity would have been induced in studies where HFD-feeding was restricted to pregnancy and lactation, or lactation alone. Therefore, these offspring likely experienced exposure to HFD in the absence of maternal obesity. It is challenging to tease out whether HFD-induced obesity before pregnancy or ongoing maternal HFD affects the skeleton in offspring using a rodent model. Unlike in humans, changing the diet of a rodent from HFD to CD induces rapid weight loss (47). This could be overcome using embryo transfer following pre-conception maternal HFD, placing embryos into CD-fed recipients (46).

Another gap in our understanding is deciphering the effects of maternal HFD on other tissues in offspring and how these effects, in turn, modulate the skeleton. For instance, there is cross-talk between the skeleton and skeletal muscle, adipose tissue, and the endocrine pancreas (48–52). The structure and function of these tissues are affected by the early life environment (7). Therefore, it would be interesting to determine whether this cross-talk is affected by maternal HFD and the downstream effects on the skeleton.

In this mini-review, we exclusively discussed the effect of maternal HFD on the skeleton in offspring; however, other paradigms of early life exposure to nutritional excess are also worthy of exploration. For instance, a maternal high-protein diet (53) and a combination of high-fat and high-sugar diet (54) also negatively impact the skeleton in offspring. Additionally, pre-conception paternal nutrition also has long-term effects on the metabolic health of offspring (55). We are unaware of any studies that have addressed the paternal influence of skeletal development in offspring. Thus, understanding the impact of paternal health will also be necessary for understanding the mechanisms that link the early life environment with skeletal health in offspring.

Critically, we are unaware of any studies investigating the effect of nutritional, pharmacological, or behavioral interventions on skeletal outcomes in the offspring. Whilst we do not yet fully understand the mechanisms that impact the developing skeleton in response to maternal HFD, a significant gap lies in the lack of intervention studies.

CONCLUSION

There is growing evidence that exposure to maternal HFD during pregnancy has long-lasting adverse effects on the skeleton of offspring. However, many details surrounding these changes and the mechanisms of action that drive these effects remain unclear, and further basic studies are required. Given the consequences of low bone mass and deranged bone microarchitecture for offspring, advances in our understanding of the developmental origins of bone health is critical in our battle against diseases like osteoporosis.

AUTHOR CONTRIBUTIONS

EB designed the review. EB, SB, and MT collected relevant articles. All authors have contributed to writing and revision of the manuscript, read, and approved the submitted version.

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A Maternal High Fat Diet Leads to Sex-Specific Programming of Mechanical Properties in Supraspinatus Tendons of Adult Rat Offspring

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Background: Over half of women of reproductive age are now overweight or obese. The impact of maternal high-fat diet (HFD) is emerging as an important factor in the development and health of musculoskeletal tissues in offspring, however there is a paucity of evidence examining its effects on tendon. Alterations in the early life environment during critical periods of tendon growth therefore have the potential to influence tendon health that cross the lifespan. We hypothesised that a maternal HFD would alter biomechanical, morphological and gene expression profiles of adult offspring rotator cuff tendon.

Materials and Methods: Female Sprague-Dawley rats were randomly assigned to either: control diet (CD; 10% kcal or 43 mg/g from fat) or HFD (45% kcal or 235 mg/g from fat) 14 days prior to mating and throughout pregnancy and lactation. Eight female and male offspring from each maternal diet group were weaned onto a standard chow diet and then culled at postnatal day 100 for tissue collection. Supraspinatus tendons were used for mechanical testing and histological assessment (cellularity, fibre organisation, nuclei shape) and tail tendons were collected for gene expression analysis.

Results: A maternal HFD increased the elasticity (Young's Modulus) in the supraspinatus tendon of male offspring. Female offspring tendon biomechanical properties were not affected by maternal HFD. Gene expression of SCX and COL1A1 were reduced in male and female offspring of maternal HFD, respectively. Despite this, tendon histological organisation were similar between maternal diet groups in both sexes.

Conclusion: An obesogenic diet during pregnancy increased tendon elasticity in male, but not female, offspring. This is the first study to demonstrate that maternal diet can modulate the biomechanical properties of offspring tendon.

A maternal HFD may be an important factor in regulating adult offspring tendon homeostasis that may predispose offspring to developing tendinopathies and adverse tendon outcomes in later life.

Keywords: tendon, animal model, rotator cuff, obesity, high-fat diet, developmental programming

INTRODUCTION

Obese and overweight individuals make up over a third of the world's population and are projected to represent an estimated 60% by 2030 (1, 2). The impact of obesity on the musculoskeletal system is emerging as an important manifestation of the disease (3, 4). Several clinical and epidemiological studies have demonstrated a negative association between obesity and tendon health (5–7). In particular, obesity is consistently a risk factor for rotator cuff tendinopathy and tendon tear (5, 8–10). In animal models, a high fat diet (HFD) has been reported to result in detrimental effects on tendon biomechanical, structural and biochemical properties (11–13).

Over half of women of reproductive age are now overweight or obese (14, 15) and approximately one in five women are obese during pregnancy (16). Adverse maternal exposures during prenatal and early postnatal life can impair offspring health across their lifespan. Maternal obesity is a risk factor for increased adiposity and glucose intolerance in adult offspring (17). In rodent pre-clinical studies, maternal obesity can be modelled using a HFD through well-established methods that has been previously demonstrate to alter maternal and offspring metabolic profiles (18–20).

A maternal HFD has a significant influence on the development and health of other musculoskeletal tissues in offspring, including bone, cartilage and muscle (21–25). Despite the increasing global prevalence of the high-fat Western diet, the effect of maternal exposure to HFD on programming of offspring tendon health is largely unknown. Alterations in the early life during critical periods of tendon development have the potential to influence offspring tendon gene, cell and tissue function throughout adult life.

The purpose of this study was to determine the effect of a maternal HFD on the tendon profile of adult male and female offspring. We hypothesised that a maternal HFD would be associated with altered biomechanical and structural properties in offspring tendon, and altered gene expression profiles of offspring tendon cells.

MATERIALS AND METHODS

Animal Model

All animal experiments were approved by the University of Auckland Animal Ethics Committee (#001936), in accordance with the New Zealand Animal Welfare Act, 1999. Reporting of *in vivo* results conforms to the ARRIVE guidelines (18). This study of tendon properties was conducted on a subset of adult offspring ($n = 8$ per group) from an unrelated larger study investigating metabolic end points in adult offspring with dietary manipulation and fish oil supplementation of pregnant rat dams ($n = 12$ per group).

Female virgin Sprague-Dawley rats were obtained at a weaning age and fed a standard chow diet (Diet 2018, Envigo Teklad Global Diets, Indianopolis, IN, USA) *ad libitum* until day 110. All animals were housed under standardised conditions at 22 ± 2°C with a 12 h light: 12 h dark cycle. At day 110, rats were then randomly assigned to either a control diet (CD; 10% kcal or 43 mg/g from fat, #D12450H, Research Diets, Diets, New Brunswick, NJ, USA) or HFD (45% kcal or 235 mg/g from fat, #D12451, Research Diets) and fed *ad libitum* for 14 days prior to mating and throughout gestation and lactation. Females were time mated using an oestrous cycle monitor (EC-40, Fine Science Tools, San Francisco, CA, USA), with pregnancy confirmed through detection of spermatozoa following vaginal lavage. Dams were continued on their study diet throughout the pregnancy and lactation (**Figure 1**).

In order to maintain standardised nutrition until weaning, litter size was randomly adjusted to eight pups per litter (4 males and 4 females) on post-natal day 2 (P2) and unused pups were euthanized. At P21, one male and one female offspring from each dam were randomly selected. Offspring were housed in sex-matched pairs and fed a standard chow diet *ad libitum* for the remainder of the study (P100). Body weights were monitored every third day. At P100, animals were fasted overnight and killed by decapitation under pentobarbitone anaesthesia (Sigma-Aldrich, St Louis, MO, USA; intraperitoneal injection; 60 mg/kg). Eight male and eight female offspring were randomly selected from each of the maternal dietary groups for tendon analysis.

From these offspring, both shoulders with the supraspinatus tendon attached to the humerus were excised immediately. The left shoulders were wrapped in a phosphate-buffered saline solution-soaked gauze and stored at −20°C for later biomechanical testing and the right shoulders were immersed in 10% neutral buffered formalin (NBF) for histological analysis. The tail was harvested and primary tenocytes were isolated from tendon fascicles for gene expression analysis. Trunk blood was collected into heparinised vacutainers (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 2,500 g at 4°C for 15 min. Plasma samples were stored at −20°C until subsequent analysis.

Plasma Analysis

Plasma was analysed for free fatty acids, triglycerides, low-density lipoprotein cholesterol (LDL), high-density lipoprotein

Abbreviations: ARRIVE, Animal research: reporting of *in vivo* experiments; CD, Control diet; cDNA, Complementary DNA; COL1A1, Collagen type I alpha 1 chain; DNA, Deoxyribonucleic acid; ECM, Extracellular matrix; HFD, High fat diet; IQR, Interquartile Range; MMP, Matrix metalloproteinase; RNA, Ribonucleic acid; ROI, Regions of interest; SCX, Scleraxis; SEM, Standard error of the mean; TNMD, Tendomodulin; 2D, Two dimensional; 3D, Three dimensional.

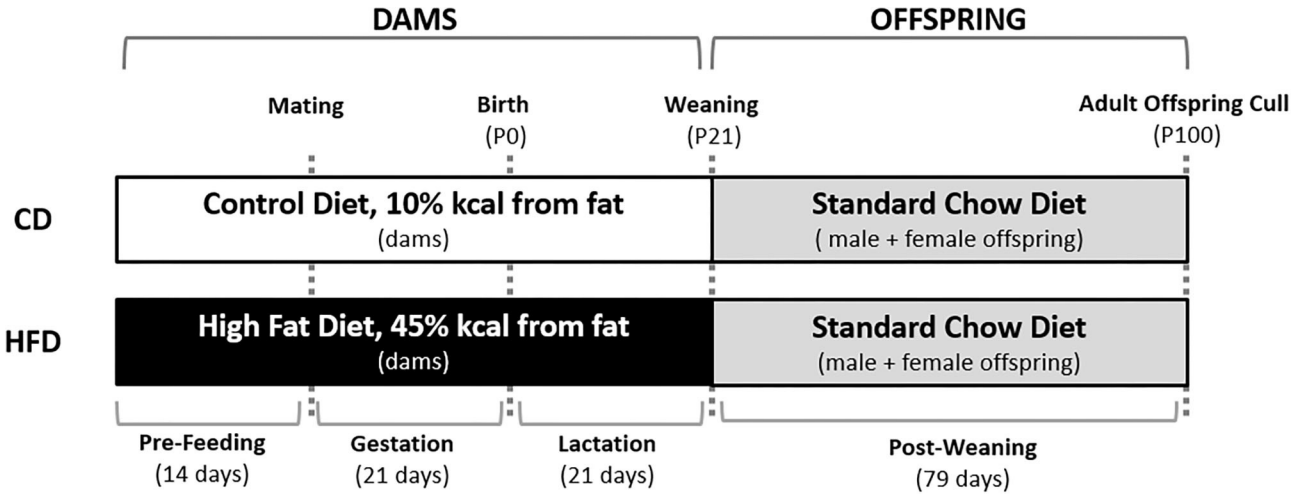

FIGURE 1 | Overview of the study design. Female Sprague-Dawley rats consumed either a CD or HFD *ad libitum* for 14 days prior to mating and throughout pregnancy and lactation. Male and female offspring were weaned onto a standard chow diet *ad libitum* at post-natal day 21 (P21). At post-natal day 100 (P100), offspring were culled and plasma and tissue was collected.

cholesterol (HDL) and total cholesterol by Hitachi 902 auto-analyser (Hitachi High Technologies Corporation, Tokyo, Japan). Plasma samples were thawed on ice and centrifuged for 3 min at 2,500 rpm at 4°C prior to performing the kits to remove fibrous clots which are common in rodent plasma. Where multiple plates were needed for one marker, samples were performed in a randomised order which was generated via an Excel database to avoid time-of-day effects and inter-assay effects. All intra- and inter- assay coefficients of variation were <5%. Plasma analysis was performed with 8 animals per group.

Biomechanical Testing

Biomechanical testing was performed using established protocols, as previously described (26). The excised shoulders were thawed at room temperature and kept hydrated with normal saline solution spray throughout testing. The supraspinatus muscle fibres were removed by gentle scraping, leaving only the distal tendon attached to the proximal humerus. The humerus and the tendon were positioned in an Instron machine (Instron, Norwood, MA, USA) with a 1-kN load cell. The tendon was secured using a double screw clamp with fine-grit sand paper, and the humerus was secured using a customised 3D printed clamp to prevent fracture through the growth plate. The width and thickness of the tendon were measured using digital callipers and cross-sectional area calculated by multiplying both measurements. The specimens were subjected to uniaxial strain in line with anatomical loading and undergo a 10-cyle precondition (0.1 to 0.5 N at rate of 0.5 mm/sec) followed by 1 min of relaxation and were then stretched to failure at a rate of 0.5 mm/s. The Young's modulus and ultimate stress at failure were recorded for each sample. Specimens mostly failed at the bone-tendon interface of the supraspinatus enthesis and only these results were included. In the three other instances, failure occurred prematurely intra-substance in the supraspinatus tendon. Therefore, 5 or 6 samples were tested for each group.

Histological Analysis

After fixing in 10% NBF for 7 days, bone-tendon-muscle units were decalcified in formic acid 10% aqueous solution for 7 days and then processed, embedded in paraffin and 7-μm-thick sections were taken. Sections were stained with haematoxylin and eosin and the tendon mid-substance was imaged at 40x magnification and viewed using both transmitted and polarised light. Cell density (number of nuclei per mm^2) and nuclear aspect ratio (the ratio of the minor diameter to the maximal diameter, with values approaching zero suggesting a spindle shape and with the value of 1.00 representing a perfect circle) were evaluated, as described in previously established protocols (27, 28). In healthy tendon, the few fibroblasts with flattened nuclei are typically aligned parallel to the tensile axis. In tendinopathy, tendon cells density increases, nuclei become more rounded, and collagen fibre alignment is disrupted (27). Six regions of interest (ROI) were measured and the mean value was taken for each sample.

The Directionality plug-in for Fiji (http://fiji.sc/Fiji, Ashburn, VA, USA) was used on polarised light images to perform 2D fast Fourier transform analysis to measure collagen fibre alignment, according to previously established methods (29, 30). The Directionality plug-in calculated the spatial frequencies within an image given a set of radial directions. The method generated normalised histograms revealing the amount of fibres present between 0° and 180° with a bin size of 1°. The plug-in then generated statistics on the highest peak found and performed a Goodness-of-fit test between the observed values and a Gaussian distribution to provide a Goodness value [0 (poorly aligned) -1 (well-aligned)]. The Goodness values were measured in six ROI's and the mean value was taken for each sample. Six samples were analysed for each group.

TABLE 1 | Physiological and metabolic profiles of each group.

	Male offspring			Female offspring		
Maternal Diet	**CD**	**HFD**	***P*-Value**	**CD**	**HFD**	***P*-Value**
Dam weight P100	314.8 (19.0)	320.8 (52.4)	0.867	314.8 (19.0)	320.8 (52.4)	0.867
Offspring weight P100 (g)	465.5 (26.6)	511.1 (66.6)	0.142	259.6 (72.0)	274.8 (61.2)	0.220
Free fatty acids (mmol/L)	0.54 (0.09)	0.67 (0.35)	0.512	0.55 (0.23)	0.66 (0.15)	0.067
Triglycerides (mmol/L)	0.69 (0.41)	0.73 (0.49)	0.662	0.74 (0.22)	0.60 (0.36)	0.377
HDL (mmol/L)	1.49 (0.66)	1.17 (0.43)	0.363	1.80 (0.45)	1.80 (0.50)	0.804
LDL (mmol/L)	0.48 (0.17)	0.37 (0.19)	0.216	0.29 (0.18)	0.28 (0.15)	0.980
Total cholesterol (mmol/L)	2.10 (0.86)	2.26 (0.70)	0.662	1.73 (0.87)	2.24 (0.70)	0.557

Data were analysed by Mann-Whitney U-test for each sex. Data are presented as median (with IQR). N = 8 per group.

Gene Expression Analysis of Tenocytes Isolated From Rat Tail Tendons

Tenocytes were isolated from tendon fascicles teased from rat tails using a previously established protocol (31). In brief, tendon was cut into <1 cm pieces and digested in 0.5 mg/ml dispase and 400 U/ml collagenase-II (both from Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's Modified Eagle's Medium: F-12 with 10% FBS at 37°C for up to 18 h until most of the extracellular matrix had been digested. The cell suspension was then passed through a cell strainer, washed, re-suspended in phosphate-buffered saline and pelleted for RNA extraction.

For analysis of gene expression, total cellular RNA was extracted from cultured cells and purified using the RNeasy minikit (Qiagen, Venlo, The Netherlands). Genomic DNA was removed using RNasefree DNase set (Qiagen). Quality and concentration of the extracted RNA was measured using NanoDrop Lite Spectrometer (Thermo-Fisher, Victoria, Australia). Complementary-DNA (cDNA) was prepared by using 300 ng of RNA with super-script-III (Life Technologies, Carlsbad, CA, USA). Primer-probe sets were purchased as TaqMan Gene Expression Assays (Life Technologies). Multiplex polymerase chain reaction was performed with FAM specific for genes of interest and VIC-labelled 18S endogenous ribosomal RNA probes, according to the manufacturer's instructions, using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Samples were assayed in triplicate. The ΔΔCt calculation method was used to determine the relative level of messenger RNA expression, normalised to the values of cells from a CD offspring for each sex. The relative gene expression of collagen type Iα1 (COL1A1), the main structural collagen present in tendon (32); scleraxis (SCX), a key transcription factor in tenocyte differentiation (33); tendomodulin (TNMD), a key glycoprotein in the proliferation and development of tenocytes (34), were determined. In addition, chondrocyte gene, SOX-9, was assessed as tenocytes tend to transdifferentiate into such cell types, one of the pathological causes of tendinopathy (35). Matrix re-modelling [matrix metalloproteinase-3 (MMP-3) and MMP-13] gene expression markers were also determined as the basal activity of MMPs is greatly modified in painful tendinopathy (36). Four samples *per sex* were analysed for each group.

Statistical Analysis

Data from the weights, blood samples, histological, biomechanical and gene expression were analysed using a Mann-Whitney *U*-test for comparison between maternal CD and maternal HFD in both male and female offspring. $P < 0.05$ was considered significant. Data are presented as median with interquartile range (IQR) and graphed using Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Maternal HFD Did Not Induce an Overt Change in Dam Body Weight and Offspring Body Weight or Metabolic Profile

At P21, there were no significant differences in the weights of dams fed a CD or HFD diet. There were no significant effects of a maternal HFD on adult offspring weights at P100 for either sex. In addition, there were no significant differences in free fatty acids, triglycerides, HDL, LDL and total cholesterol in either male or female offspring with maternal HFD (**Table 1**).

A Maternal HFD Impacted Tendon Elasticity in Adult Male Offspring, but Did Not Affect Tendon Biomechanical Properties in Female Offspring

In male offspring, the Young's modulus was significantly higher with maternal HFD compared to maternal CD [29.72 (IQR 28.94) vs. 59.23 (IQR 15.85), $P = 0.030$]. There was no significant effect of maternal HFD in female offspring [37.71 (IQR 24.17) vs. 41.78 (IQR 22.62), $P = 0.222$]. There was no significant difference in stress of failure and cross-sectional area between HFD CD offspring groups (**Table 2**).

TABLE 2 | Supraspinatus tendon biomechanical properties.

	Male offspring			Female offspring		
Maternal Diet	**CD**	**HFD**	***P*-Value**	**CD**	**HFD**	***P*-Value**
Cross-sectional area (cm^2)	1.17 (0.04)	1.16 (0.25)	0.695	1.08 (0.16)	1.30 (0.26)	0.159
Young's modulus (MPa)	29.72 (28.94)	**59.23 (15.85)***	**0.030**	37.71 (24.17)	41.78 (22.62)	0.222
Stress at failure (MPa)	20.52 (13.33)	27.54 (7.40)	0.429	16.71 (5.26)	19.55 (11.84)	0.222

*Data were analysed by Mann-Whitney U-test for each sex, where *P < 0.05 compared to CD offspring. Data are presented as median (with IQR). N = 6 per group. Values in bold are significantly different (P < 0.05) compared to maternal CD.*

TABLE 3 | Detailed histologic scores.

	Male offspring			Female offspring		
Maternal Diet	**CD**	**HFD**	***P*-Value**	**CD**	**HFD**	***P*-Value**
Cellularity (cells per mm^2)	1,101 (329.7)	1,067 (326.5)	0.937	1,413 (336.0)	1,359 (301.0)	0.574
Nuclei Circularity (0–1)	0.425 (0.236)	0.497 (0.182)	0.485	0.524 (0.061)	0.476 (0.149)	0.126
Directionality Goodness Value (0–1)	0.926 (0.056)	0.883 (0.073)	0.071	0.920 (0.073)	0.914 (0.050)	0.662

Data were analysed by Mann-Whitney U-test for each sex. Data are presented as median (with IQR). N = 6 per group.

Offspring Tendon Histological Structure Was Not Affected With Maternal HFD

A maternal HFD had no significant effect on cell density or nuclei shape in either male or female offspring, compared to maternal CD (**Table 3**; **Figure 2**). A maternal HFD also did not significantly affect tendon collagen organisation (Directionality Goodness value) in either sex (**Table 3**). Averaged normalised histograms demonstrating the similar distribution of collagen fibre alignment for each sex and maternal diet are shown in **Figure 3**.

Lower Gene Expression of Scleraxis in Male Offspring and Collagen Type Iα1 in Female Offspring of Dams Fed a HFD

In primary tenocytes of the male, but not female, offspring of maternal HFD, there was significantly lower expression of tenocytic marker SCX gene (−33%, $P = 0.029$). In female, but not male, offspring of maternal HFD there was lower expression of tenocytic COL1A1 gene (−71%, $P = 0.029$) (**Figure 4**). There was a strong trend in reduction of chondrocytic maker SOX-9 in female offspring of dams fed HFD, however this did not reach statistical significant ($P = 0.061$). Maternal diet had no significant effect on the gene expression levels for other tenocyctic (TNMD), chrondrocytic (SOX-9) or matrix re-modelling (MMP-3 and MMP-13) markers in either male or female offspring.

DISCUSSION

Here, we have demonstrated for the first time that maternal diet can influence offspring tendons. A maternal diet high in fat increased the Young's modulus of the supraspinatus tendon of male offspring, and altered the gene expression profile of tendon cells from offspring of both sexes. However, maternal HFD did not significantly alter the histological structural properties of supraspinatus tendon in male or female offspring.

A maternal obesogenic diet has been associated with a range of adverse effects across several physiological systems (37–39). Emerging pre-clinical and clinical evidence suggests that maternal exposure to HFD may have long-term consequences on the musculoskeletal system in offspring (40–45). A maternal HFD has been shown to increase the size of type 1 and 2A fibres in skeletal muscle, promoting a more oxidative profile, and elicit lifelong mitochondrial alterations (46–48). In bone, maternal HFD has been demonstrated to negatively impact osteoblast performance and lead to osteopenia in adult offspring, independent of post-weaning diet (44, 45). Foetal pre-osteoblastic cell senescence signalling appears to be epigenetically regulated by maternal obesity to repress bone formation in adult offspring (23).

Previous studies have reported direct detrimental effects of consuming a HFD on the biomechanical properties of rodent tendon (11–13). Interestingly, these tendinopathic changes do not seem to resolve with dietary intervention from a HFD to low-fat diet, suggesting that any pathologic change induced in tendon with HFD is irreversible (49). Previous studies of other musculoskeletal tissues, including muscle and bone, have shown that post-weaning exposure to HFD has an additive deleterious effect with maternal HFD exposure on programmed diseased tissue phenotypes (23, 47). We therefore may have seen more pronounced effects on tendon properties with exposure to a post-weaning HFD in offspring. Future studies should investigate the possible additive effects of maternal HFD and the offspring ageing on adverse tendon outcomes.

We found sex-specific effects of a maternal HFD in alteration of offspring tendon properties, with only male tendon elasticity reduced at P100. Sex-specific variability in tendon biomechanical properties is well-established and thought to be the result of

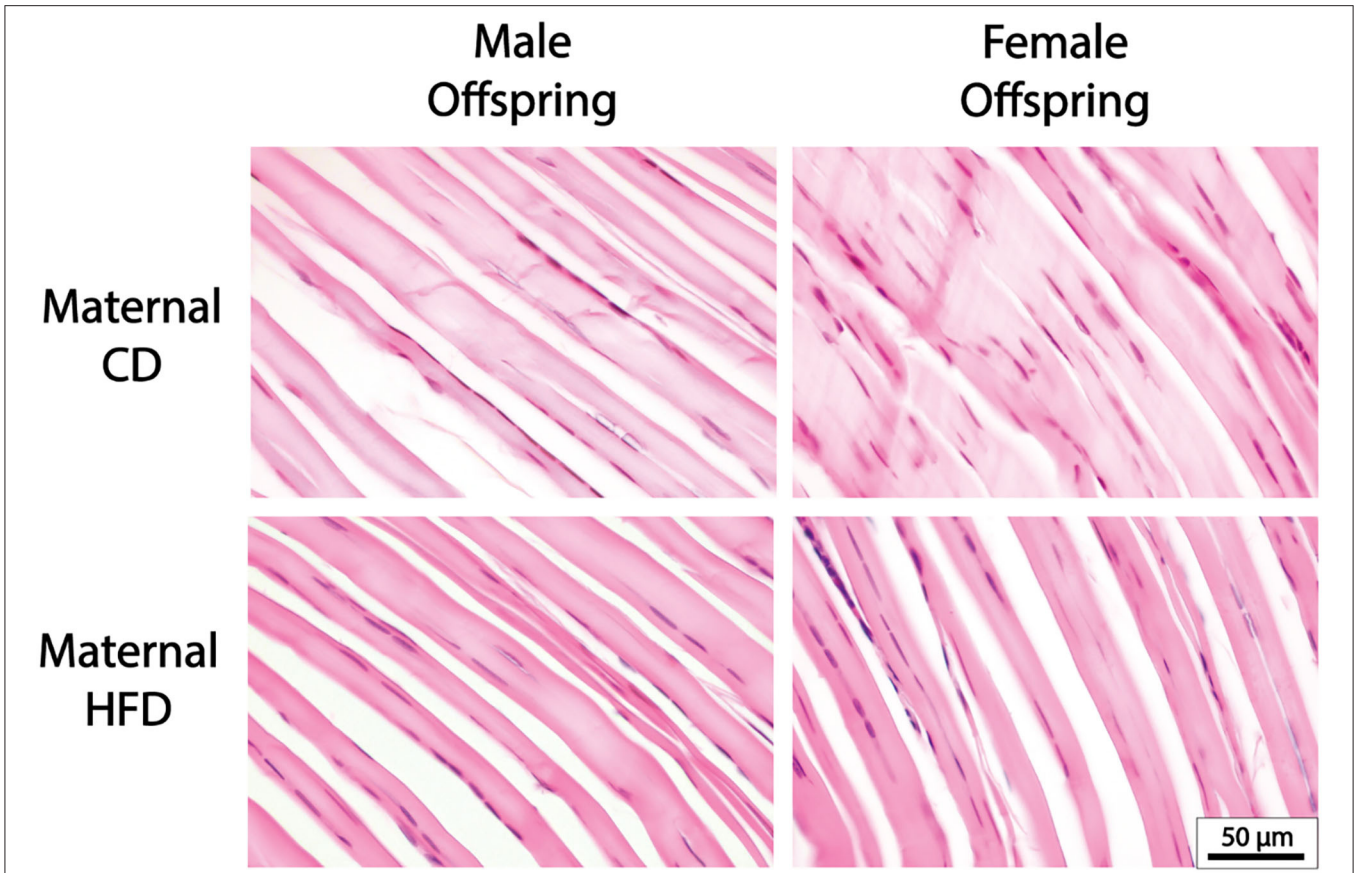

FIGURE 2 | Representative Tendon Histology Images. There were no significant differences in cellularity, collagen alignment or nuclei shape with maternal HFD in either male or female offspring.

hormonal influence (50–53). Elevated levels of oestradiol reduce collagen synthesis in ligaments and tendons, and thus collagen remodelling may be less pronounced in females. This could be reducing the responsiveness of female offspring tendon to the effects of maternal HFD (52, 53). Another possibility is that sex-specific differences in the underlying epigenetic regulation of genes associated with tendon development could be contributing to this variation.

In this study there was lower expression of tenocytic-marker SCX gene in male, but not female, offspring of a maternal HFD. Scleraxis is a basic helix-loop-helix transcription factor and plays a central role during embryonic tendon development. SCX gene expression drives matrix production and re-modelling, epithelial-to-mesenchymal transition, development of force-transmitting tendon and tendon growth (54–57). Scleraxis is required for mechanically stimulated adult tendon growth through driving the expression of extracellular matrix (ECM) components, so changes in SCX expression could have diverse impact on offspring tendon development (56, 57). In female, but not male, offspring of a maternal HFD, there was reduced of expression of type I collagen gene. Collagen type Iα1 accounts for the majority of dry weight of tendon ECM. A major focus of tenocyte metabolism in post-natal tendon tissue is to maintain the ECM integrity by regulating type I collagen production (58, 59). This is the first evidence that maternal HFD can influence gene expression in adult offspring and requires more research to determine the regulatory pathways which may be altering their expression.

Determining the mechanisms by which a maternal HFD diet results in altered tendon properties will require additional study. Previous studies have suggested that effect of obesity on tendon may be secondary to alterations with circulating cholesterol and lipid levels (11, 13). It has been proposed that elevated cholesterol levels may alter the tendon microenvironment via local changes in protein synthesis and extracellular matrix composition/turnover (60). In this study, there were no differences in the lipid and cholesterol profiles of offspring animals with a maternal HFD. This suggests that effects on offspring tendon are occurring independently of hypercholesterolemia. There is a growing understanding of epigenetic control in the onset and progression of other musculoskeletal diseases, including osteoarthritis and osteoporosis (25, 61, 62). There is evidence of epigenetic regulation in the expression of genes specifically associated with tendon development, including scleraxis, collagen type Iα1 and tendomodulin (63). Other studies have also shown that maternal and paternal epigenetic modifications play essential roles in the development of tendon (64–66). Thus, we speculate

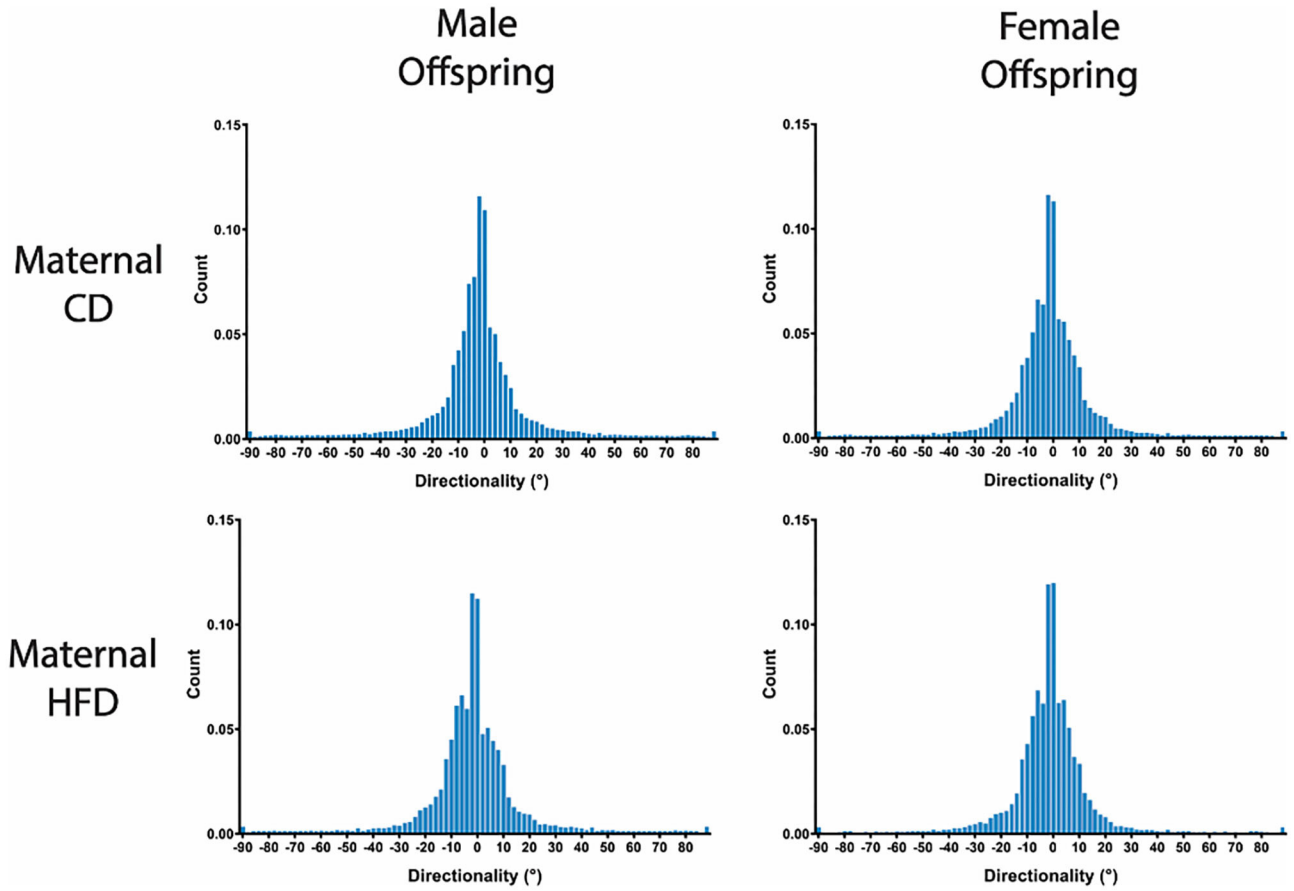

FIGURE 3 | Group averaged Directionality Histograms of Collagen Fibre Alignment. Averaged histograms demonstrating the distribution of collagen fibres present between 0° and 180° with a bin size of 1° for each sex and maternal diet. *N* = 6 per group.

the maternal diet could alter epigenetic histone modifications, DNA methylation, and non-coding RNAs in offspring to confer susceptibility to tendinopathy and tendon rupture that persists throughout life.

LIMITATIONS

The rats in this experiment were a relatively young age of 14 weeks old (P100) when tissue was collected. Ageing is a known risk factor in the onset of tendinopathy and therefore a longer study period may have resulted in more significant changes in tendon properties. Similarly, more significant changes may have been observed following injury to the tendons, with a maternal HFD potentially predisposing the tendons to poorer healing outcomes. Therefore, future research could consider not only the effects of maternal diet on tendon health, but also tendon injury and healing.

In this study we only included a sub-set of animals from a large study for assessment of tendon properties and it is possible higher animal numbers may have identified further differences. However, the sample size chosen for tendon analysis was based on previous experimental studies (n = 6–9 per group) investigating the effect of post-weaning HFD exposure on biomechanical and histological properties (11, 12, 67).

Here, we used supraspinatus tendon to explore the biomechanical and histological effects of maternal HFD, and rat tail tendon to explore changes in tenocyte gene expression profiles. It would have been optimal to explore all outcomes from the same tendon origin, as this would allow for associations to be made between the gene and tissue level changes. However, the supraspinatus tendon is not suitable for obtaining RNA/cells to carry out such studies due to its small size and acellular nature. The rat tail, however, is a well-validated source of tenocytes and represents a good model of tendon cell behaviour (68–72). While we were unable to correlate gene level changes with tissue level alterations, individually these findings are novel and provide valuable information about the effect of maternal high fat diet on offspring tendon properties.

Furthermore, changes in tenocyte behaviour were determined at the gene expression level from RNA extracted from cell pellets digested direct from the tail tendon fascicles. The whole cell pellet was used for this purpose, to ensure there was sufficient extracted RNA. This prevented the gene expression changes observed being validated with corresponding protein level analysis, which will be important to look at in future studies.

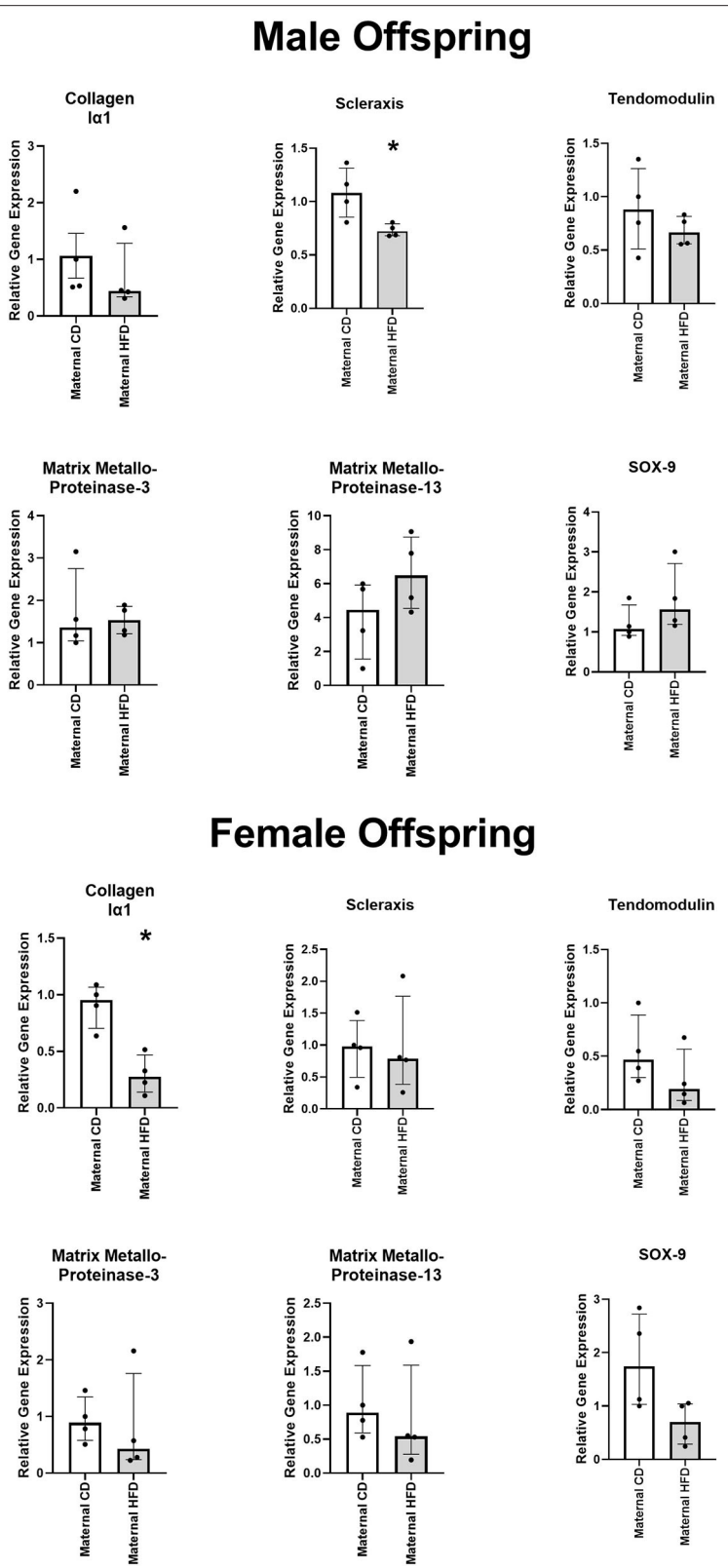

FIGURE 4 | Effect of maternal HFD on the gene expression profile of primary tenocytes derived from tail tendon. Data were analysed by Mann-Whitney *U*-test for each sex. Data are presented as median (with IQR), where $^*P < 0.05$ compared to CD offspring. $N = 4$ per group.

The length of maternal exposure to HFD was relatively short in this study, and although it included the entirety of pregnancy and lactation, the dietary intervention began only 14 days prior to mating. We may have observed more pronounced effects on offspring tendon phenotype with longer duration of pre-conception maternal HFD. The lack of an overt programmed metabolic phenotype in our offspring may also be a result of the control diet utilised. The current study utilised a matched semi-purified control diet in the dams, previous work has utilised a standard chow-based diet and the differences in energy/caloric intake between these control diets may explain the differences in phenotypes observed (18). Programming effects in offspring can be subtle when fed a standard diet post-natally and only amplified in the setting of a post-weaning HFD (23, 47). Thus, further studies could examine the effects of maternal HFD alone and combination with a postnatal HFD in offspring.

Finally, this study was conducted in a rodent model, and although maternal HFD during pregnancy is a well-established model of offspring obesity independent of post-natal diet, there are discrepancies in gene expression alterations between obese rats and humans which could potentially limit the applicability to humans (73).

CONCLUSIONS

This is the first study to demonstrate that maternal diet influences tendon homeostasis and biomechanical properties in adult offspring. This research suggests that maternal HFD may be an important factor in regulating an offspring tendon phenotype that predisposes adult offspring to adverse tendon outcomes and higher prevalence of tendon injury in adult life.

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 University of Auckland Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

SB participated in study design, acquisition of data, interpretation of data, drafting of the article, and revision of the manuscript. VS participated in study design, acquisition of data, interpretation of data, and revision of the manuscript. SK participated in acquisition of data, drafting of the article, and revision of the manuscript. BC, AM, and JC participated in analysis, interpretation of data, and revision of the manuscript. JM participated in study design, analysis, interpretation of data, and revision of the manuscript. MV participated in study design, acquisition of data, analysis, interpretation of data, and revision of the manuscript. BA participated in study design, acquisition of data, analysis, and revision of the manuscript. DM participated in study design, acquisition of data, analysis, drafting of the article, and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Association of Maternal Dietary Patterns With Birth Weight and the Mediation of Gestational Weight Gain: A Prospective Birth Cohort

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The associations among maternal diet, birth weight, and gestational weight gain are still inconclusive. This study aimed to investigate the associations between maternal dietary patterns and birth weight, and further explore whether GWG mediates these associations. A total of 3,334 pregnant women who completed a validated semi-quantitative food frequency questionnaire from the Tongji Maternal and Child Health Cohort were included. Dietary patterns were extracted by using principal component analysis. Regression models and mediation analyses were performed to explore the associations between dietary patterns and birth weight and the effects of GWG on these associations. Five dietary patterns were identified: "Beans-vegetables," "Fish-meat-eggs," "Nuts-whole grains," "Organ-poultry-seafood" and "Rice-wheat-fruits." Only women following the "Beans-vegetables" pattern had heavier newborns (β = 47.39; 95% CI: 12.25, 82.54). Women following the "Beans-vegetables" pattern had significantly lower GWG (β = −0.7; 95% CI: −1.15, −0.25) and had a 16% lower risk of excessive GWG and 11% higher odd of adequate GWG. The association between the "Beans-vegetables" pattern and birth weight was negatively mediated by GWG. A dietary pattern enriched in beans and vegetables is beneficial for effectively controlling GWG and increasing birth weight. GWG serves.

Clinical Trial Registry: This trial was registered at ClinicalTrials.gov (NCT03099837).

Keywords: dietary patterns, birth weight (BW), gestational weight gain (GWG), mediation analysis, plant-based foods, pregnant population

INTRODUCTION

Birth weight is an important index to evaluate the health status of newborns. Inappropriate birth weight is related to an increased risk of infant mortality and diseases in adulthood life (1–4). Therefore, correcting suboptimal birth weight could provide newborns with both short-term and long-term health benefits.

Nutrition plays a crucial role in the growth and development of a fetus. Several studies focusing on specific nutrients and foods have advanced our comprehension of the links between maternal nutrition and birth weight (5). In practice, it has a bias to figure out the exact effect of a single factor due to the interplay between nutrients and foods in the process of digestion and absorption in the human body. Moreover, the specific nutrient deficiency diseases have evolved into chronic conditions which were associated with imbalanced diets over the past few decades (6). To address these, the dietary pattern is applied to characterize the diet on the whole and evaluate its association with birth weight.

It is concordance across many studies that optimal birth weight was associated with what were perceived as healthier diets, such as the Mediterranean diet and the Dietary Approaches to Stop Hypertension diet, which was rich in vegetables, beans, and seafood, among other (7, 8). The pattern rich in vegetables and fruits was associated with lower birth weight in the Norwegian population, whereas women who followed similar diets were more likely to have heavier babies in multiethnic Asian and Chinese populations (9–11). Thus, the relationship between dietary patterns and birth weight is still inconclusive.

Furthermore, gestational weight gain (GWG) of mothers is associated with the birth weight of newborns (12). A meta-analysis revealed that the proportion of excessive GWG was up to 47% according to the Institute of Medicine criteria in 2009 (12). Excessive GWG triggers adverse birth outcomes such as fetal overgrowth and obesity and metabolic dysfunction of offspring for long-term (13, 14). In the context of the association among maternal dietary patterns, GWG and birth weight, we hypothesized that maternal dietary patterns may affect birth weight in two ways: directly or indirectly (mediated by GWG). Therefore, the objective of this study is to figure out the associations between maternal dietary patterns and birth weight, and further explore whether GWG mediates these associations from a large, prospective cohort of pregnant women in central China.

MATERIALS AND METHODS

Study Design

The Tongji Maternal and Child Health Cohort (TMCHC) is a prospective cohort in Wuhan, Hubei province, central China, to investigate the associations between maternal dietary, lifestyle factors, and the pregnant outcomes of mothers and newborns from January 2013 to May 2016. Pregnant women at 8–16 weeks of gestation were enrolled in this cohort. This was the time when they went to the hospital for their first antenatal visit in one of three public hospitals in Wuhan. At enrollment, all participants completed an interviewer-administrated questionnaire which included some baseline information. Trained investigators conducted lifestyle and dietary intake interviews during each trimester. This study was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Review Committee of Tongji Medical College, Huazhong University of Science and Technology. All participants gave informed written consent upon recruitment.

The subjects who were included in this analysis had completed the food frequency questionnaire (FFQ) and been sure to undergo regular prenatal examination and delivery in the above hospitals. Those who reported a previous gestational diabetes mellitus (GDM) or history of diabetes ($n = 86$), multiple gestations ($n = 135$), an implausible total energy intake (<600 or >3,500 kcal/day) ($n = 81$), who had abortion or stillbirth ($n = 22$), or who did not have information about the diagnosis of GDM ($n = 364$) were excluded in this study. Finally, a total of 3,334 women were available (**Supplementary Figure 1**).

Dietary Intake Assessment

The dietary intake assessment was obtained with a validated FFQ during the past 4 weeks and all participants completed FFQ during the second trimester of a pregnancy before the diagnosis of GDM (15). This FFQ consisted of food type, frequency of intake, and average consumption per serving of each food in the past 4 weeks (28 days), containing 61 food items and 16 non-overlapping food groups, covering more than 200 kinds of foods (16). We adjusted the 15 categories of frequency into six grades ("never," "1–3 times per 4 weeks," "1–3 times per week," "4–6 times per week," "1–2 times per day" and "more than two times per day"). The daily intake of each food was calculated by multiplying the number of servings per 4 weeks by the average consumption per serving and then dividing by 28. According to the quantity of energy and nutrient per 100 g of different foods in the China Food Composition Database, energy and nutrient intakes of some food were calculated by this content per 100 g multiplied by daily intake of this food (17). Then we could acquire the daily energy and nutrient intakes by adding up all food intakes. The formulas were as follows:

$$NC(unit/d) = \sum_{i=1}^{n} \frac{Mi \times Ni \times Pi}{28 \times 100}$$

Where NC: average daily intake of energy or some nutrient; i: a variety of foods; M: average consumption per serving; N: numbers of servings per 4 weeks; P: quantity of some nutrient in this type of food per 100 g.

Outcomes Assessments

Gestational weight gain during pregnancy was defined as the difference between the last available weight measurement during pregnancy and the pre-pregnancy weight. The last available weight measurement was measured by investigators on admission to the hospital while awaiting delivery. The pre-pregnancy weight was self-reported using a questionnaire at the time of enrollment during their first antenatal visit to the hospital. Birth weight, birth length, and sex of newborns were obtained through medical records. Some important definitions included: normal birth weight referred to the neonatal birth weight ≥2,500 and <4,000 g; the ponderal index (PI) was calculated as birth weight (kg)/birth length^3 (m^3); the newborns were defined as large for gestational age (LGA) when the body weight was >90th percentile for gestational age and small for gestational age (SGA) when it was <10th percentile for gestational age; excessive GWG,

adequate GWG, and insufficient GWG were evaluated based on the recommendation of Institute of Medicine (18).

Covariates Assessment

The demographic and socioeconomic characteristics, anthropometric parameters, and lifestyle of the participants were recorded using a structured questionnaire at the baseline enrollment. The questionnaire was completed by trained investigators, including the information of maternal age, educational level, average personal income, ethnicity, pre-pregnancy weight, height, history of the disease, history of the family disease, parity, physical activity, consumption of smoke and alcohol, and so on. Maternal age was categorized as ≤24, 25–29, 30–35, and ≥36 years old. Ethnicity was categorized as Han Chinese and others. Education level was divided into ≤9 (junior high school or under), 10–12 (senior high school or technical secondary school), 13–15 (bachelor or college degree), and ≥16 (master or above) years (19). Per capita monthly income was divided into five categories: ≤1,000, 1,001–2,999, 3,000–4,999, 5,000–9,999, and ≥ 10,000 CNY. Pre-pregnancy body mass index (BMI) was divided into four categories according to the BMI classification criteria suitable for Chinese people: <18.5, 18.5–23.9, 24–27.9, and ≥28 kg/m^2. Smoking status was divided into "yes (either smoking or passive smoking)" and "no (none)" according to whether smoking was a habit before pregnancy, or whether participants were frequently exposed to second-hand smoke (more than 15 min a day and more than 3 days a week, lasting half a year). Alcohol consumption was divided into "yes" and "no" categories based on whether alcohol was consumed before pregnancy (more than 3 days a week, lasting half a year). History of disease included chronic non-communicable diseases, infectious disease, and hereditary disease. History of a family disease considered these diseases of immediate families (parents and siblings). The history of specific diseases was considered as binary variables, divided into "yes" and "no".

Statistical Analysis

Dietary patterns were extracted by principal component analysis. This analysis was a data-driven technique that reduced the dimensions of the data and grouped correlated variables to identify common factors/components (i.e., dietary patterns) by varimax rotation. The principle of principal component analysis was to decompose the total variance of the original indicators into the sum of the variances of several independent composite factors. The first principal component contributed the most to explaining total variation. The greater the contribution of variation among the factors, the better their ability to combine the original indicators. So, the factors represented the combinations of food consumed by individual participants. The number of factors retained was based on the eigenvalues, the breakpoints of the scree plot, cumulative variances, and factor interpretability. Thereinto, the factor loading reflected the relevance between the original 16 non-overlapping food group frequency and newly extracted factors (dietary patterns). In this study, the food groups with factor loadings of 0.40 or higher on a factor were considered important to the interpretability of each pattern. As the patterns were extracted, each participant had the dietary pattern score corresponding to each dietary pattern. The score was calculated by summing the mean standardized frequency of food groups weighted by their factor loadings. Higher dietary pattern scores indicated greater adherence to the extracted patterns (10, 20).

We described the covariates and dietary characteristics by using ANOVA or independent sample *t*-tests. To explore the associations between dietary pattern scores and pregnant outcomes, we treated dietary pattern scores as quartiles and as continuous variables. Multivariate linear regression models were used to analyze the relations between dietary pattern scores and continuous pregnant outcomes, such as GWG and birth weight. In addition, the changes in pregnancy outcomes and *P*-value were calculated when the dietary pattern score increased by one unit. For the relations between scores and binary pregnant outcomes, such as excessive GWG, adequate GWG, insufficient GWG, SGA, and LGA, Logistic regression models were used. Stratified analyses were conducted by sex of newborns. Mediation analysis was an approach to assess the importance of various pathways and mechanisms, which had expanded over the past decade (21). We used mediation analysis to explore the effect of GWG on the associations between dietary patterns and birth weight. It was performed using bootstrapping, and Model 4 was run in Process (V3.2) with covariates in SPSS (22). All statistical analyses were performed using the SPSS software version 22 (IBM Corp., Armonk, NY). A two-sided α of less than 0.05 was considered statistically significant.

RESULTS

Dietary Patterns and Pregnant Outcomes

Overall, 3,334 participants were included in this analysis. Five dietary patterns were identified (**Table 1**), and we named them "Beans-vegetables," "Fish-meat-eggs," "Nuts-whole grains," "Organ-poultry-seafood" and "Rice-wheat-fruits." The average age was 28.12 years, and the average pre-pregnancy BMI was 20.77 kg/m^2. Almost all participants were Han Chinese (97.0%). Other social and demographic characteristics of participants were present in **Supplementary Table 1**. The scores of each pattern had significant differences in maternal educational level, average personal income, age, parity, and so on, as displayed in **Supplementary Table 2**.

Pregnant outcomes of all participants were presented in **Supplementary Table 3**. The average GWG was 15.87 ± 4.49 kg and the average birth weight of newborns was 3338.19 ± 448.46 g. For mothers, excessive, adequate, and insufficient GWG made up 44.9, 33.3, and 21.8%, respectively. For newborns, SGA, and LGA accounted for 7.3 and 8.2%, respectively.

Dietary Patterns in Relation to Normal Birth Weight and Other Birth Outcomes

Whether unadjusted or adjusted, the relationships between dietary pattern scores and normal birth weight only existed in the "Beans-vegetables" pattern, as displayed in **Table 2**. After adjusted covariates, the newborn birth weight of the highest quartile increased 47.39 g (95% CI: 12.25, 82.54; $P_{trend} = 0.012$). Considering the score as a continuous variable, normal birth weight increased by 17.58 g (95% CI: 5.18, 29.98; $P = 0.005$) with

TABLE 1 | Factor loading matrix for dietary patterns identified by principal component analysis ($n = 3{,}334$)[a].

Food groups[b]	Dietary patterns				
	Beans-vegetables	**Fish-meat-eggs**	**Nuts-whole grains**	**Organ-poultry-seafood**	**Rice-wheat-fruits**
Root vegetables	0.70	—	—	—	—
Mushrooms and algae	0.58	—	—	—	—
Melon and solanaceous vegetables	0.57	—	—	—	—
Beans and bean products[c]	0.54	—	—	—	—
Leafy and cruciferous vegetables	0.46	—	—	—	—
Red meat	—	0.61	—	—	—
Freshwater fishes	—	0.59	—	—	—
Eggs	—	0.58	—	—	—
Nuts	—	—	0.70	—	—
Whole grains	—	—	0.48	—	—
Dairy products[d]	—	—	0.48	—	—
Animal organ and blood	—	—	—	0.64	—
Seafood	—	—	—	0.64	—
Poultry	—	—	—	0.58	—
Rice and wheat products	—	—	—	—	0.84
Fruits	—	—	—	—	0.51
Cumulative variance explained (%)[e]	11.4	20.7	29.6	38.3	45.1

[a] *Values were factor loadings (correlation coefficients) between each food frequency variable and the dietary pattern derived from principal component analysis.*
[b] *Food groups were sorted by the size of loading coefficients. Absolute values <0.4 were not listed for simplicity.*
[c] *Denoted soybean, mung bean, soybean milk, bean curd, and so on.*
[d] *Denoted milk, milk powder, and yogurt.*
[e] *Percentage of variance in total food intake explained by patterns.*

one unit increase in this score. Although "the Fish-meat-eggs" pattern score had no significant relationship with birth weight, the risk of LGA increased 18% as the score increased by one unit (95% CI: 1.04, 1.34). Other associations between dietary pattern scores and birth outcomes were exhibited in **Table 3**.

After stratified analysis according to the offspring sex, the relation of "Beans-vegetables" only appeared in boys. Compared to the lowest quartile, the birth weight of the highest quartile increased 56.79 g (95% CI: 7.8, 105.78; $P_{\text{trend}} = 0.024$). One unit increase in dietary pattern score was associated with the increase of 20.41 g of birth weight of boys (95% CI: 2.91, 37.91, $P = 0.022$).

The contributions of nutrients on the association between "Beans-vegetables" patterns and normal birth weight were explored. For total newborns, the association of "Beans-vegetables" pattern score and birth weight was not attenuated substantially after adjusting various nutrients. However, for boys, the association between "Beans-vegetables" pattern score and birth weight for comparisons of highest with lowest quartiles was no longer significant after further adjustment for plant protein ($\beta = 50.66$; 95% CI: −1.72, 103.04), fiber ($\beta = 50.88$; 95% CI: −3.36, 105.11), total iron ($\beta = 47.65$; 95% CI: −4.68, 99.98) and non-heme iron ($\beta = 49.79$; 95% CI: −2.5, 102.09), which indicated that these nutrients contributed to the association between "Beans-vegetables" pattern score and birth weight (**Supplementary Table 4**).

Dietary Patterns in Relation to GWG

Associations between the degree of adherence to the dietary patterns and GWG were exhibited in **Table 4**. Except for the "Organ-poultry-seafood" pattern, the dietary pattern scores of the other four patterns were either significantly correlated or inversely correlated with GWG. Compared to the lowest quartile of "Beans-vegetables" patterns, the GWG of the highest quartile reduced 0.7 kg (95% CI: −1.15, −0.25; $P_{\text{trend}} = 0.002$). With a unit of "Beans-vegetables" pattern score increased, GWG decreased 0.36 kg (95% CI: −0.52, −0.20; $P < 0.001$). In addition, this pattern reduced the 16% risk of excessive GWG and increased the 11% likelihood of adequate GWG (95% CI: 0.77, 0.91 and 95% CI: 1.03, 1.21, respectively), while it increased 14% risk of insufficient GWG in the meantime (95% CI: 1.03, 1.26). For the other three patterns, the GWG of the highest quartile all showed an increase compared with the lowest quartile. GWG increased 0.18 kg for "Fish-meat-eggs" (95% CI: 0.01, 0.34), 0.23 kg for "Nuts-whole grains" (95% CI: 0.07, 0.39) and 0.26 kg for "Rice-wheat-fruits" (95% CI: 0.04, 0.48) as score increased by one unit. And the risks of insufficient GWG for these three patterns decreased 15, 13, and 17%. Moreover, the "Rice-wheat-fruits" pattern increased the 14% likelihood of GWG in the appropriate range (95% CI: 1.02, 1.26) (**Table 3**).

Mediation of GWG in the Relationship Between "Beans-Vegetables" Pattern and Normal Birth Weight

Mediation analysis was performed to clarify the role of GWG in the relationship between the "Beans-vegetables" pattern and normal birth weight, as displayed in **Table 5**. We found that the relationship between the "Beans-vegetables" pattern and birth weight was negatively mediated by GWG ($\beta_{\text{indirect}} = -4.50$; 95%

TABLE 2 | Associations between dietary pattern scores and normal birth weight (g) (n =2,847)[a].

Dietary patterns	Quartiles of dietary pattern scores				*P* for trend	Per unit increase	*P*-value
	Q1	Q2	Q3	Q4			
Beans-vegetables							
Mean (min, max)	−1.30 (−3.89–0.65)	−0.29 (−0.65, 0.05)	0.37 (0.05, 0.71)	1.22 (0.72, 3.11)			
Model 1	Reference	12.27 (−22.84, 47.34)[b]	13.81 (−21.69, 49.31)	45.40 (10.17, 80.63)	0.015	17.69 (5.27, 30.11)	0.005
Model 2	Reference	12.22 (−22.88, 47.31)	13.53 (−21.99, 49.05)	44.91 (9.66, 80.16)	0.016	17.65 (5.23, 30.08)	0.005
Model 3	Reference	12.97 (−22.05, 27.99)	12.73 (−22.63, 48.10)	47.39 (12.25, 82.54)	0.012	17.58 (5.18, 29.98)	0.005
Boy	Reference	2.26 (−46.91 ,51.43)	12.62 (−36.42 ,61.66)	56.79 (7.80,105.78)	0.024	20.41 (2.91, 37.91)	0.022
Girl	Reference	23.89 (−25.68 ,73.47)	5.5 (−45.40,56.4)	34.63 (−15.78 ,85.05)	0.271	12.80 (−4.70, 30.30)	0.152
Fish-meat-eggs							
Mean (min, max)	−1.32 (−4.36, −0.60)	−0.24 (−0.60, 0.07)	0.37 (0.07, 0.68)	1.19 (0.68, 3.12)			
Model 1	Reference	13.52 (−21.74, 48.78)	30.74 (−4.50, 65.97)	15.51 (−19.75, 50.76)	0.263	4.85 (−7.58, 17.28)	0.444
Model 2	Reference	10.08 (−25.28, 45.44)	27.19 (−8.19; 62.56)	15.06 (−20.24, 50.37)	0.275	4.93 (−7.52, 17.37)	0.438
Model 3	Reference	7.37 (−27.88, 42.63)	29.68 (0–5.67, 65.03)	0.39 (−36.00, 36.79)	0.669	1.07 (−11.94, 14.07)	0.842
Boy	Reference	−8.63 (−58.25, 41.00)	35.85 (−14.25, 85.95)	0.62 (−49.79, 51.02)	0.560	4.71 (−13.49, 22.91)	0.612
Girl	Reference	21.13 (−28.92, 71.17)	23.52 (−26.07, 73.12)	−7.54 (−60.21, 45.14)	0.858	−1.29 (−19.81, 17.23)	0.892
Nuts-whole grains							
Mean (min, max)	−1.34 (−5.26, −0.65)	−0.25 (−0.65, 0.10)	0.41 (0.10, 0.72)	1.18 (0.72, 2.75)			
Model 1	Reference	42.03 (6.65, 77.40)	18.41 (−16.73, 53.54)	35.29 (0.29, 70.29)	0.142	11.21 (−1.30, 23.73)	0.079
Model 2	Reference	40.35 (4.84, 75.86)	15.25 (−20.14, 50.65)	35.24 (0.25, 70.23)	0.151	11.16 (−1.35, 23.68)	0.080
Model 3	Reference	37.40 (2.06, 72.75)	2.20 (−32.98, 37.38)	18.44 (−17.31, 54.19)	0.732	4.16 (−8.63, 16.95)	0.524
Boy	Reference	27.07 (−21.66, 75.81)	−11.13 (−60.36, 38.10)	7.61 (−43.04, 58.27)	0.840	2.26 (−16.10, 20.63)	0.809
Girl	Reference	47.44 (−4.18, 99.05)	23.88 (−26.35, 74.10)	38.06 (−12.26, 88.37)	0.259	9.13 (−8.80, 27.06)	0.318
Organ-poultry-seafood							
Mean (min, max)	−1.25 (−2.56, −0.73)	−0.37 (−0.73, −0.05)	0.30 (−0.04, 0.66)	1.31 (0.66, 3.78)			
Model 1	Reference	−8.44 (−43.86, 26.97)	16.65 (−18.70, 51.99)	−0.17 (−35.53, 35.20)	0.664	2.38 (−10.10, 14.85)	0.709
Model 2	Reference	−8.07 (−43.50, 27.35)	18.35 (−17.00, 53.70)	−0.04 (−35.38, 35.31)	0.642	2.51 (−9.96, 14.97)	0.693
Model 3	Reference	−7.14 (−42.24, 27.97)	24.96 (−10.08, 60.00)	1.84 (−33.56, 37.25)	0.495	4.71 (−7.79, 17.20)	0.460
Boy	Reference	−21.82 (−70.58, 26.94)	24.00 (−25.18, 73.18)	3.75 (−45.40, 52.90)	0.477	8.09 (−9.18, 25.36)	0.358
Girl	Reference	11.20 (−39.34, 61.74)	32.12 (−17.66, 81.89)	0.22 (−50.82, 51.27)	0.785	0.93 (−17.35, 19.21)	0.920
Rice-wheat-fruits							
Mean (min, max)	−1.01 (−21.54, −0.23)	−0.01 (−0.23, 0.18)	0.33 (0.18, 0.47)	0.69 (0.47, 1.67)			
Model 1	Reference	8.05 (−27.21, 43.31)	8.45 (−26.67, 43.56)	0.44 (−34.81, 35.68)	0.973	4.49 (−11.41, 20.39)	0.580
Model 2	Reference	−0.71 (−36.64, 35.23)	−1.12 (−37.05, 34.80)	0.12 (−35.19, 35.43)	0.999	4.25 (−11.64, 20.14)	0.600
Model 3	Reference	−6.52 (−42.38, 29.34)	0.84 (−35.06, 36.74)	−2.17 (−37.71, 33.38)	0.986	6.76 (−9.53, 23.05)	0.416
Boy	Reference	−14.70 (−64.99, 35.60)	−20.39 (−70.41, 29.64)	−19.82 (−70.36, 30.72)	0.401	7.60 (−17.17, 32.36)	0.548
Girl	Reference	−2.12 (−53.23, 48.99)	20.19 (−31.61, 71.99)	19.80 (−30.29, 69.88)	0.319	2.95 (−18.54, 24.44)	0.788

[a]*Multivariate linear regression models were as followed: Model 1, crude model; Model 2, adjusted for other four dietary patterns; Model 3, adjusted for Model 2 + maternal age, physical activity, ethnology, maternal education, average personal income, family history of diabetes, family history of obesity, smoking habit, alcohol habit, parity, pre-pregnancy BMI, GDM, gestational weight gain, and total energy intake.*

[b]*Values were presented as β (95% CI) (all such values).*

CI: −7.27, −2.04). After adjusted covariates, this mediation was still significant with an estimated mediating proportion of −26% ($\beta_{\text{indirect}} = -4.67$; 95% CI: −7.69, −1.87). Besides, this mediating effect also remained in newborns of boys, and the estimated mediating proportion was −21.3% ($\beta_{\text{indirect}} = -4.44$; 95% CI: −8.68, −0.41).

DISCUSSION

We extracted five dietary patterns to represent the dietary habits of pregnant women in central China, naming them as "Beans-vegetables," "Fish-meat-eggs," "Nuts-whole grains," "Organ-poultry-seafood," and "Rice-wheat-fruits," respectively. Women who tended to have higher adherence to the "Beans-vegetables" pattern had infants with relatively high birth weight. Higher adherence to the "Beans-vegetables" pattern was significantly associated with the decrease of GWG, the reduction of excessive GWG risk, and the increase of adequate GWG odd. The association between the "Beans-vegetables" pattern and birth weight was partly mediated by GWG.

TABLE 3 | Associations between maternal dietary pattern scores and pregnant outcomes[a].

Pregnant outcomes	Dietary patterns				
	Beans-vegetables	Fish-meat-eggs	Nuts-whole grains	Organ-poultry-seafood	Rice-wheat-fruits
GWG (kg)[b]	−0.36 (-0.52,−0.20)**	0.18 (0.01, 0.34)*	0.23 (0.07, 0.39)*	−0.01 (-0.17, 0.15)	0.26 (0.04, 0.48)*
Birth weight (g)[b]	32.25 (10.40, 54.11)*	2.94 (-18.93, 24.79)	3.79 (-18.23, 25.80)	4.30 (-16.46, 25.05)	6.92 (-22.87, 36.71)
Normal birth weight (g)[b]	17.58 (5.18, 29.98)*	1.07 (-11.94, 14.07)	4.16 (-8.63, 16.95)	4.71 (-7.79, 17.20)	6.76 (-9.53, 23.05)
Birth length (cm)[b]	0.09 (0.01, 0.16)*	−0.04 (-0.11, 0.04)	0.00 (-0.07, 0.08)	−0.02 (-0.09, 0.05)	0.02 (-0.12, 0.09)
Ponderal index (kg/m^3)[b]	0.15 (0.04, 0.26)*	0.07 (-0.04, 0.18)	0.01 (-0.10, 0.12)	0.06 (-0.41, 0.17)	0.06 (-0.09, 0.21)
Excessive GWG[c]	0.84 (0.77, 0.91)**	1.07 (0.99. 1.16)	1.07 (0.99, 1.15)	0.94 (0.87, 1.01)	1.01 (0.92, 1.12)
Adequate GWG[c]	1.11 (1.03, 1.21)*	1.04 (0.96, 1.13)	1.02 (0.94, 1.11)	1.04 (0.96, 1.12)	1.14 (1.02, 1.26)*
Insufficient GWG	1.14 (1.03, 1.26)*	0.85 (0.77, 0.95)*	0.87 (0.79, 0.96)*	1.06 (0.96, 1.17)	0.83 (0.74, 0.93)*
SGA[c]	0.89 (0.78, 1.02)	0.97 (0.85, 1.10)	0.95 (0.83, 1.08)	0.98 (0.87, 1.11)	0.97 (0.86, 1.10)
LGA[c]	1.04 (0.92, 1.18)	1.18 (1.04, 1.34)*	1.00 (0.88, 1.13)	1.07 (0.95, 1.21)	1.11 (0.98, 1.26)

[a]*Multivariate linear regression and multinomial logistic regression models were used. Models were adjusted for maternal age, physical activity, ethnology, maternal education, average personal income, family history of diabetes, family history of obesity, smoking habit, alcohol habit, parity, pre-pregnancy BMI, gestational diabetes mellitus status, gestational weight gain, infant sex, total energy intake, and other four dietary patterns. LGA, large for gestational age; SGA, small for gestational age; GWG, gestational weight gain.*
[b]*Values were presented as β (95% CI) for continuous variables.*
[c]*Values were presented as OR (95% CI) for categorical variables.*
*$P < 0.05$. **$P < 0.001$.*

TABLE 4 | Associations between dietary pattern scores and gestational weight gain (kg) ($n = 3{,}110$)[a].

Dietary patterns	Quartiles of dietary pattern scores				*P* for trend	Per unit increase	*P*-value
	Q1	Q2	Q3	Q4			
Beans-vegetables							
Mean (min, max)	−1.30 (−3.89, −0.65)	−0.29 (−0.65, 0.05)	0.37 (0.05, 0.71)	1.22 (0.72, 3.11)			
Model 1	Reference	−0.05 (−0.50, 0.39)[b]	−0.30 (−0.75, 0.15)	−0.60 (−1.05, −0.15)	0.005	−0.31 (−0.47, −0.15)	<0.001
Model 2	Reference	−0.05 (−0.49, 0.39)	−0.34 (−0.78, 0.11)	−0.65 (−1.10, −0.21)	0.002	−0.32 (−0.48, −0.17)	<0.001
Model 3	Reference	−0.13 (−0.57, 0.31)	−0.35 (−0.79, 0.10)	−0.70 (−1.15, −0.25)	0.002	−0.36 (−0.52, −0.20)	<0.001
Fish-meat-eggs							
Mean (min, max)	−1.32 (−4.36, −0.60)	−0.24 (−0.60, 0.07)	0.37 (0.07, 0.68)	1.19 (0.68, 3.12)			
Model 1	Reference	0.19 (−0.26, 0.64)	0.10 (−0.35, 0.54)	0.62 (0.17, 1.06)	0.015	0.20 (0.04, 0.36)	0.013
Model 2	Reference	0.25 (−0.20, 0.70)	0.15 (−0.30, 0.59)	0.71 (0.26, 1.15)	0.005	0.23 (0.07, 0.39)	0.004
Model 3	Reference	0.16 (−0.30, 0.60)	0.02 (−0.13, 0.47)	0.60 (0.14, 1.05)	0.028	0.18 (0.01, 0.34)	0.035
Nuts-whole grains							
Mean (min, max)	−1.34 (−5.26, −0.65)	−0.25 (−0.65, 0.10)	0.41 (0.10, 0.72)	1.18 (0.72, 2.75)			
Model 1	Reference	0.32 (−0.13, 0.77)	0.66 (0.21, 1.10)	0.65 (0.21, 1.10)	0.001	0.26 (0.10, 0.42)	0.001
Model 2	Reference	0.22 (−0.23, 0.67)	0.54 (0.10, 0.99)	0.65 (0.20, 1.09)	0.002	0.25 (0.09, 0.41)	0.002
Model 3	Reference	0.10 (−0.34, 0.55)	0.45 (0.08, 0.90)	0.55 (0.10, 1.01)	0.006	0.23 (0.07, 0.39)	0.006
Organ-poultry-seafood							
Mean (min, max)	−1.25 (−2.56, −0.73)	−0.37 (−0.73, −0.05)	0.30 (−0.04, 0.66)	1.31 (0.66, 3.78)			
Model 1	Reference	−0.25 (−0.70, 0.20)	−0.17 (−0.61, 0.28)	−0.04 (−0.49, 0.41)	0.979	−0.01 (−0.16, 0.15)	0.945
Model 2	Reference	−0.21 (−0.65, 0.24)	−0.14 (−0.58, 0.31)	−0.06 (−0.50, 0.39)	0.886	−0.01 (−0.17, 0.15)	0.890
Model 3	Reference	−0.18 (−0.62, 0.27)	−0.13 (−0.57, 0.31)	0.04 (−0.44, 0.45)	0.929	−0.01 (−0.17, 0.15)	0.934
Rice-wheat-fruits							
Mean (min, max)	−0.89 (−6.51, −0.23)	−0.01 (−0.23, 0.18)	0.33 (0.18, 0.47)	0.69 (0.47, 1.67)			
Model 1	Reference	0.78 (0.33, 1.22)	1.05 (0.60, 1.49)	1.14 (0.70, 1.58)	<0.001	0.43 (0.22, 0.64)	<0.001
Model 2	Reference	0.80 (0.35, 1.25)	1.06 (0.60, 1.51)	1.20 (0.76, 1.64)	<0.001	0.44 (0.22, 0.65)	<0.001
Model 3	Reference	0.58 (0.13, 1.04)	0.69 (0.24, 1.15)	0.76 (0.32, 1.21)	<0.001	0.26 (0.04, 0.48)	0.021

[a]*Multivariate linear regression models were as followed: Model 1, crude model; Model 2, adjusted for other four dietary patterns; Model 3, adjusted for Model 2 + maternal age, physical activity, ethnology, maternal education, average personal income, family history of diabetes, family history of obesity, smoking habit, alcohol habit, parity, pre-pregnancy BMI, GDM, and total energy intake.*
[b]*Values were presented as β (95% CI) (all such values).*

TABLE 5 | Mediating effects of gestational weight gain (kg) (n = 2,793)[a].

Variables	Direct effect	Indirect effect	Estimated percent mediated (%)
Model 1	22.99 (10.68, 35.30)[b]	−4.50 (−7.27, −2.04)	−24.3
Model 2	23.17 (10.84, 35.49)	−4.70 (−7.49, −2.31)	−25.6
Model 3	22.64 (10.44, 34.84)	−4.67 (−7.69, −1.87)	−26.0
Boy	25.29 (8.05, 42.53)	−4.44 (−8.68, −0.41)	−21.3

[a]*Mediation analyses were used. Models were as followed: Model 1, crude model; Model 2, adjusted for other four dietary patterns; Model 3, adjusted for Model 2 + maternal age, physical activity, ethnology, maternal education, average personal income, family history of diabetes, family history of obesity, smoking habit, alcohol habit, parity, pre-pregnancy BMI, gestational diabetes mellitus, gestational weight gain, and total energy intake.*
[b]*Values were presented as β (95% CI) (all such values).*

In this study, we observed that the "Beans-vegetables" pattern contributed to increasing birth weight in the normal range, without generating the risk of LGA. Consistent with our results, some studies came to the conclusion that maternal dietary patterns rich in plant foods were associated with larger birth sizes in multiethnic Asian and Chinese populations (10, 11). The study of Zulyniak et al. also proved among South Asians living in Canada that a plant-based diet was associated with higher birth weight but not associated with the risk of having an SGA or LGA newborns (23). The "Beans-vegetables" pattern identified in the present study also decreased GWG and controlled it in an optimal range. Similar results existed in other studies. Women from a mother-offspring cohort in Singapore who tended to consume similar plant-based foods had a decreased risk for inadequate GWG and excessive GWG (24). Beyond that, in a prospective cohort of Brazilian pregnant women, the "common-Brazilian" pattern rich in beans had no association with excessive GWG and was positively associated with adiponectin, which had characteristics of anti-obesity and anti-inflammation (25). Thus, a dietary pattern enriched in beans and vegetables has a beneficial effect on optimal birth weight and GWG.

Previous studies have found a positive link between GWG of women and the birth weight of newborns, and GWG above the recommendations was associated with a higher risk of LGA (12, 26, 27). Lu et al. and Wei et al. also found that the effect of a specific dietary pattern on GWG of pregnant women and the birth weight of newborns coexisted in the Chinese Guangzhou population (11, 28). To figure out the potential function of GWG, we further performed a mediation analysis to clarify the role of GWG in the relationship between the "Beans-vegetables" pattern and birth weight. As mentioned above, considering GWG, both the total effect and the direct effect of the "Beans-vegetables" pattern were positive on birth weight, while the indirect effect was negative. As a result, this indirect effect weakened the total effect. GWG may serve as a mediator in the association of the "Beans-vegetables" pattern and birth weight. That means, on one hand, the "Beans-vegetables" pattern itself could directly promote the increase of birth weight, on the other hand, it may also indirectly prevent excess birth weight by controlling GWG.

Our further investigation revealed that plant protein, fiber, and iron served as contributors to the association between the "Beans-vegetables" pattern and birth weight, which may explain the potential role of plant-based foods. It is worth mentioning the beneficial effect of plant protein in plant-based foods. There have been few studies that show a direct effect of maternal plant protein intake on birth weight. However, Lai et al. found a positive association between plant protein-enriched foods and optimal GWG (24). The results of another prospective study among adults who participated in the Diet, Genes, and Obesity (Diogenes) project also showed that the source but not the amount consumed was related to weight gain (29). Specifically, sources of protein that vary in amino acid composition should be considered here. Plant protein enhances insulin sensitivity and energy expenditure due to low in branched-chain amino acids (BCAAs) and sulfur-containing amino acids (SCAAs), which may interpret the association between plant protein and weight gain (30–32). Aside from plant protein, other characteristics of plant-based foods, including relatively low energy density and high fiber, merit attention. They play vital roles in appetite regulation, metabolism, and tissue maintenance in order to maintain a normal weight (24). On the contrary, animal-based foods are characterized as high cholesterol and saturated fatty acids (SFAs), both of which have been shown to be associated with an increased risk of obesity in previous studies (33, 34). Certainly, more strictly designed animal and human experiments are needed to further explore the specific mechanisms of the effect of plant-based food components on birth weight, either directly or indirectly *via* GWG.

The findings of the beneficial effect of the "Beans-vegetables" pattern only appeared in boys in the present study. This may be explained by a sex-specific growth mechanism during the fetal period. Generally, boys have higher birth weights, possibly due to an interaction between sex hormones, fetal insulin, and genetic factors (35). Available evidence suggested the sex-specific adaptation of the placenta may be the core of the differences in fetal growth (36). The placentas of male and female fetuses have different protein, gene expressions, instituting different mechanisms to cope with an adverse intrauterine environment or event, which is reflected in their birth weight (37–39). As for one of the focuses of intrauterine environment–maternal diet, it is proposed that boys are more sensitive and dependent on maternal diet during pregnancy, making them able to capitalize on improving food supply but vulnerable to food shortage (40, 41). It is because of this sensitive growth strategy of boys that warns of the need to pay more attention to intrauterine nutrition to improve birth outcomes.

The predominant advantage of this study is the use of prospective design to clarify the association between maternal dietary patterns and birth weight. Moreover, we took the mediation of GWG into consideration and analyzed direct and indirect effects of dietary patterns, respectively when exploring their relationship with birth weight. However, we acknowledge some limitations of this study. First, the dietary data of this study was obtained from a single FFQ investigation for 24–28 weeks. But as a previous study suggested, a single source of dietary data can provide reliable information throughout pregnancy (42). Second, using self-reported pre-pregnancy weight would introduce bias into GWG measurements. However, this is a practical and cost-effective method, and no matter in published literature or the sensitivity analysis of a previous study on the

same population, the results revealed that the bias appeared to be small (43–45). Last, potential dietary and non-dietary confounders were adjusted in the analysis, while the possibility of residual confounding from unmeasured or unknown covariates could not be ruled out.

CONCLUSIONS

In conclusion, this prospective cohort study in pregnant women in central China has shown that adherence to the "Beans-vegetables" pattern, which is characterized by high vegetables and beans intake, is beneficial for effectively controlling GWG in mothers and moderately increasing birth weight in newborns. In addition, GWG serves as a mediator in the association between this dietary pattern and birth weight, helping to control birth weight in a normal range. These novel findings may provide guidance for pregnant women to adhere to a healthy diet for beneficial pregnant outcomes. Well-designed intervention studies are needed to confirm our findings and elucidate the metabolic mechanisms underlying these findings.

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 studies involving human participants were reviewed and approved by Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and XZ: conceived and designed the research, acquired the data, performed the statistical analysis, drafted the manuscript, and played an important role in interpreting the results. YZ, CZ, LHu, XC, RC, JW, QL, GS, HY, GX, LHa, and NY: acquired the data. XY: revised the manuscript and further contributed to the discussion. All authors read and approved the final vision of 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/fnut.2021.782011/full#supplementary-material

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Impact of Maternal Intake of Artificial Sweetener, Acesulfame-K, on Metabolic and Reproductive Health Outcomes in Male and Female Mouse Offspring

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Guidelines advising pregnant women to avoid food and beverages with high fat and sugar have led to an increase in the consumption of "diet" options sweetened by artificial sweeteners (AS). Yet, there is limited information regarding the impact of AS intake during pregnancy on the long-term risk of cardiometabolic and reproductive complications in adult offspring. This study examined the influence of maternal acesulfame-K (Ace-K) and fructose consumption on metabolic and reproductive outcomes in offspring. Pregnant C57BL/6 mice received standard chow *ad-libitum* with either water (CD), fructose (Fr; 20% kcal intake), or AS (AS; 12.5 mM Ace-K) throughout pregnancy and lactation (n = 8/group). Postweaning offspring were maintained on a CD diet for the remainder of the experiment. Body weight, food intake, and water intake were measured weekly. Oral glucose tolerance tests (OGTT) were undertaken at 12 weeks, and the offspring were culled at week 14. Female, but not male, AS groups exhibited decreased glucose tolerance compared to Fr. There was an increase in gonadal fat adipocyte size in male offspring from AS and Fr groups compared to CD groups. In female offspring, adipocyte size was increased in the Fr group compared to the CD group. In female, but not male offspring, there was a trend toward increase in *Fasn* gene expression in AS group compared to the CD group. Maternal AS and Fr also negatively impacted upon female offspring estrus cycles and induced alterations to markers associated with ovulation. In summary, exposure to Ace-k *via* the maternal diet leads to impaired glucose tolerance and impacts adipocyte size in a sex-specific manner as well as significantly affecting estrus cycles and related gene markers in female offspring. This has implications in terms of providing tailored dietary advice for pregnant women and highlights the potential negative influence of artificial sweetener intake in the context of intergenerational impacts.

Keywords: artificial sweetener, metabolic health, reproductive, maternal nutrition, adipose tissue

INTRODUCTION

The consumption of artificial sweeteners (AS) has increased in recent decades (1, 2), with beverages being a common mode of intake. This increase is partly due to the rising awareness regarding the link between sugar intake and the risk for obesity and other associated non-communicable diseases. However, despite the promoted beneficial effects of AS, a number of studies have suggested that AS consumption can elevate the risk of insulin resistance (IR), type 2 diabetes mellitus (T2DM), obesity, and cardiovascular disease (3–6), and negatively impact the reproductive system (7, 8). It is now well established that suboptimal conditions during pregnancy and lactation, including poor maternal nutrition, can have an impact on the risk for development of disorders in offspring, including cardiometabolic disease, obesity (9), and reproductive disorders such as polycystic ovarian syndrome (PCOS) or anovulation in later life (10, 11). In this context, AS consumption may influence the health of offspring due to negative impacts on development *in utero* and during the early neonatal period, although there remains a paucity of data in this area.

Pregnant women are frequent consumers of AS, with over a quarter (29.5%) of pregnant women reported consuming AS during pregnancy, of which over 5% do so daily (12, 13). In women diagnosed with gestational diabetes mellitus (GDM), just under half (45.4%) report AS intake during pregnancy, and 9.2% report daily intake (14). AS intake during pregnancy increases the risk of preterm birth as observed in both human and mouse studies (13, 15), while AS such as saccharin, sucralose, and acesulfame-K (Ace-K) have been found in breast milk samples from women nursing infants, indicating that these chemicals may then be passed on to the developing infant during lactation (16, 17). Evidence from human cohorts also implicates maternal AS consumption in the increased incidence of obesity in offspring at one (12), and 7 years of age (14). Further studies in mice have shown the presence of Ace-K in the amniotic fluid and the mother's milk following intraoral feeding, which directly increases male offspring's sweet-taste preference for Ace-K in adulthood (18). Previous work from this group in mice has also demonstrated that maternal AS and fructose (Fr) intake induces maternal metabolic dysfunction and negatively alters fetal development in mice (15).

However, despite growing evidence that suggests adverse impacts of AS consumption, the regulations and guidelines relating to AS intake, in particular during pregnancy, remain unclear (19). The Academy of Nutrition and Dietetics, for instance, deems consumption of AS during pregnancy acceptable (20). Contradictory evidence from studies indicating no significant metabolic impairments following consumption (21) confounds confusion around the safety of AS, particularly during pregnancy and lactation. Further, differences between types of AS highlight the need for comprehensive studies into those AS most prevalent in our diets, such as Ace-K, which is one of the most popular AS (22). In human studies, differences in findings may be due to various confounding factors such as interactions with other dietary intakes and the overall dietary pattern which makes delineation of the direct effects of AS difficult. Animal models are therefore a key tool for understanding the potential effects of AS intake due to the ability to more tightly control potential experimental confounders. However, there remains a paucity of information regarding the effects of a maternal diet of AS in relation to the metabolic and reproductive health of offspring in experimental animal models, despite the growing need for such information. The aim of this study was therefore to determine the influence of the AS, Ace-K, on metabolic markers in male and female offspring, and estrus cyclicity and related ovarian gene expression in females following maternal dietary exposures during pregnancy and lactation.

METHODS

Animal Procedures

All animal procedures were approved by the Animal Ethics Committee at the University of Auckland (Approval number 001846) in accordance with the New Zealand Animal Welfare Act, 1999. Breeding pairs of C57BL/6 mice were purchased from the Vernon Jansen Unit at the University of Auckland and housed under standard conditions (wood shavings as bedding, 22°C, 40–45% humidity, and a 12-h light−12-h dark cycle).

All mice were maintained on a standard chow diet (Envigo, 2018 Teklad Global 18% Protein Rodent Diet, Ind, USA) *ad-libitum* throughout the experiment. Age-matched female mice were housed with an unrelated C57BL/6 male for mating at 10 weeks of age. Confirmation of pregnancy by vaginal plug was recorded as gestational day (GD) 0.5. Pregnant dams were then randomly assigned onto one of the following three dietary groups ($n = 8$/group):

a) Control (CD; standard diet and drinking water)
b) Artificial sweetener (AS; standard diet and 12.5 mM Ace-K in drinking water)
c) Fructose (Fr; standard diet and 34.7 mM fructose in drinking water)

Artificial sweetener and Fr doses were calculated to be equivalent to a human dose of one standard can of soda a day. Diets were maintained throughout pregnancy and lactation. Date of birth was noted and marked as postnatal day 1 (P1). At postnatal day 2 (P2), litters were weighed, sex was determined using anogenital distance, and litters were randomly reduced to eight pups per litter (four males and four females) to standardize nutrition until weaning. Offspring were weaned at postnatal day 21 and one male and one female from each litter were assessed to represent true biological replicates across the eight litters generated per dietary group. Food and water intake were also measured weekly. All offspring were fed the standard control diet and water *ad-libitum* from weaning until the end of the experiment (14 weeks). An oral glucose tolerance test (OGTT) was carried out 2 weeks prior to cull. At 14 weeks of age, mice were fasted for 6 h and tail blood and plasma samples were collected as detailed below. Mice were then weighed and culled by cervical dislocation. Female mice were staged prior to cull to ensure all female offspring were in diestrus. Gonadal adipose tissue and, in female offspring, ovaries were dissected, weighed, and snap-frozen, subcutaneous

fat was collected and snap-frozen, and then stored at −80°C for later analysis.

Measurement of Pubertal Onset

Onset of puberty in female offspring was determined by sighting of vaginal canalization. Screening for puberty onset began at day 25, occurring every morning at a consistent time (0900h), and continued until vaginal canalization was observed. The age and body weights of offspring at the time of puberty onset were recorded.

Estrus Staging

Estrus cycle staging was carried out through vaginal smear cytology, as detailed previously (23). Smearing began 5 weeks prior to cull to ensure a minimum of 5–6 cycles per animal. Smearing occurred at the same time every morning (0900h), using soft-tipped cotton buds dipped in clean saline solution (0.9%). Slides were stained by hematoxylin baths and the relative abundance of leukocytes, nucleated vaginal epithelial cells, and cornified epithelial cells in each smear were examined using light microscopy. Cycles were deemed regular if they exhibited the typical 4-day cycle, and irregular if a stage was extended or did not follow the regular pattern. Persistent estrus was determined by two or more days of estrus over one or more cycles.

Oral Glucose Tolerance Test

At 12 weeks of age, mice were fasted for 6 h from 8 a.m. Following weighing, the tip of the tail was snipped (<1 mm) and the second drop of blood was read using a glucometer (Accu-Chek Performa, Roche Diabetes Care, Ind, USA). Mice received 2 g/kg D-glucose *via* oral gavage. Blood glucose concentrations were measured from the tail tip at 0, 15, 30, 60, 90, and 120 min. Tail blood was also collected into EDTA microvettes (CB300, Sarstedt, NC, USA) at 0, 15, and 60 min. Samples were centrifuged for 10 min/1500 x *g*/4°C, and the resulting plasma was stored at −20°C for plasma insulin analysis.

Histological Analysis

Gonadal and subcutaneous adipose tissues were fixed in 10% neutral-buffered formalin, then paraffin embedded and sectioned (10 μm) using a Leica R 2,135 rotary microtome (Leica Instruments, Singapore). The slides underwent H&E staining and were then mounted with DPX mountant. Slides were visualized at 20x and images were captured using a light microscope and NIS Elements-D software (Nikon 800). Four representative images of each section were taken and analyzed in a blinded manner with ImageJ software (NIH) to determine the mean adipocyte size and distribution.

Plasma Analysis

The mouse-specific insulin, leptin, and testosterone ELISA kits (Ultra Sensitive Mouse Insulin ELISA Kit (Cat. # 90080), the Mouse Leptin ELISA kit (Cat. # 90030), and the Mouse Testosterone ELISA Kit (Cat. # 80552), Crystal Chem Inc., IL, USA) were used according to the manufacturer's instructions.

Gene Expression Analysis

Female ovarian RNA was extracted using RNeasy Mini Kits (Cat. No 74104, Qiagen, Hilden, Germany) and a TissueLyser (Qiagen, Hilden, Germany) as per the manufacturers' instructions.

Adipose tissue RNA was extracted using the trizol extraction method and a TissueLyser (Qiagen, Hilden, Germany). Following homogenization, the lysate was centrifuged for 10 min/1200 x *g*/4°C, and the accumulated fat layer was removed to improve RNA yield. Following the addition of isopropanol, samples were incubated overnight at −20°C.

Following extraction, all RNA were assessed with a NanoDrop spectrophotometer (NanoPhotometer N60, Implen). A High Capacity cDNA Reverse Transcription Kit (Life Technologies Ltd., Applied Biosystems, MS, USA) was used to generate cDNA as per the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). Genes were normalized to the geomean of *Rps29* and *Rps13* expression. The comparative CT method was utilized to analyze the results (24).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism and IBM SPSS Statistics Data Editor Version 27. The Shapiro–Wilk test was used to assess normality. Any data that was not normally distributed were transformed as appropriate. Female and male offspring data were analyzed separately. Repeated measures two-way ANOVA was performed for the OGTT data. Estrus cyclicity data were analyzed using nominal logistic regression. All other data were analyzed using the one-way ANOVA. The Holm–Sidak *post-hoc* tests were performed as indicated for comparison testing between the groups. Significance between the groups was considered at $P < 0.05$. Unless otherwise stated, all data are presented as mean ± SEM with an $n = 8$ per sex per maternal dietary group.

RESULTS

Body Weight and Caloric Intake

Maternal AS and Fr consumption had no impact on offspring birth, weaning, or final cull weight in either female or male offspring. Similarly, there was no difference in absolute gonadal fat weight or when expressed relative to body weight in either female or male offspring. There was no effect of maternal dietary group on ovary or testes weights. There was no difference between groups in relation to male/female ratios and litter size (**Table 1**).

Plasma Analysis

Plasma leptin and insulin concentrations and HOMA-IR index were unchanged by either maternal AS or Fr consumption in female and male mice. There was no change in fasting plasma glucose concentrations at cull in female offspring. However, in male mice, AS reduced glucose concentrations compared to both CD and Fr groups. Maternal diet had no effect on the testosterone concentrations in female offspring. However, in male mice, maternal Fr intake significantly increased the plasma testosterone concentrations compared to CD and AS groups (**Table 2**).

TABLE 1 | Birth, weaning, and cull weight, and gonads and gonadal fat weight of female and male offspring.

	CD	AS	Fr	Main effect ($P < 0.05$)
Females				
Birth weight (g)	1.38 ± 0.06	1.30 ± 0.03	1.36 ± 0.02	NS
Weaning weight (g)	8.06 ± 0.62	7.86 ± 0.56	7.74 ± 0.71	NS
Cull weight (g)	20.07 ± 0.39	19.52 ± 0.27	20.21 ± 0.35	NS
Ovaries (g)	0.012 ± 0.003	0.013 ± 0.003	0.013 ± 0.003	NS
Ovaries (% BW)	0.060 ± 0.003	0.061 ± 0.004	0.059 ± 0.003	NS
Gonadal fat (g)	0.32 ± 0.13	0.30 ± 0.14	0.31 ± 0.11	NS
Gonadal fat (% BW)	1.48 ± 0.14	1.37 ± 0.14	1.45 ± 0.11	NS
Pubertal onset (day)	30.67 ± 0.54	31.35 ± 0.71	32.25 ± 0.64	NS
Weight at pubertal onset (g)	14.42 ± 0.3	14.57 ± 0.3	15.12 ± 0.4	NS
Males				
Birth weight (g)	1.43 ± 0.05	1.40 ± 0.03	1.36 ± 0.03	NS
Weaning weight (g)	8.30 ± 0.26	8.23 ± 0.42	7.65 ± 0.26	NS
Cull weight (g)	26.28 ± 0.45	26.01 ± 0.26	25.57 ± 0.75	NS
Testes (g)	0.21 ± 0.014	0.21 ± 0.023	0.21 ± 0.011	NS
Testes (% BW)	0.77 ± 0.014	0.78 ± 0.019	0.77 ± 0.017	NS
Gonadal fat (g)	0.44 ± 0.17	0.46 ± 0.15	0.43 ± 0.12	NS
Gonadal fat (% BW)	1.55 ± 0.14	1.71 ± 0.13	1.55 ± 0.11	NS
Male/Female Ratio	0.91 ± 0.16	1.18 ± 0.23	1.02 ± 0.19	NS
Litter size	7.88 ± 0.48	8.38 ± 0.46	8.25 ± 0.25	NS

Puberty onset and weight at pubertal onset of female offspring, male/female ratio, and litter size. Data presented as mean ± SEM. Birth and weaning weights represent eight litters per group. For all other measures n = 8 per sex per group. NS, Not significant.

TABLE 2 | Plasma biochemical analysis in female and male offspring.

	CD	AS	Fr	Main effect ($P < 0.05$)
Females				
Leptin (ng/ml)	1.49±0.20	1.56±0.25	1.63±0.20	NS
Insulin (ng/ml)	0.47±0.049	0.39±0.041	0.43±0.044	NS
Glucose (mmol/l)	8.82±0.22	8.44±0.18	8.34±0.29	NS
HOMA-IR	1.89±0.18	1.52±0.17	1.63±0.17	NS
Testosterone (ng/ml)	0.37±0.074	0.51±0.099	0.47±0.094	NS
Males				
Leptin (ng/ml)	1.57±0.48	1.22±0.20	0.85±0.18	NS
Insulin (ng/ml)	0.75±0.064	0.79±0.065	0.81±0.078	NS
Glucose (mmol/l)	9.22±0.19	8.35±0.22*	9.49±0.25^{+}	0.002
HOMA-IR	3.18±0.33	3.12±0.27	3.53±0.32	NS
Testosterone (ng/ml)	1.92±0.83	0.90±0.29	8.92±2.67^{*+}	0.002

*Data are presented as mean ± SEM, where *p < 0.05 w.r.t CD, +p < 0.05 w.r.t AS; n = 8 per sex per group. NS, Not significant. HOMA, fasting insulin (microU/L) × fasting glucose (nmol/L)/22.5.*

Oral Glucose Tolerance Tests

In female offspring, glucose tolerance in Fr offspring was improved compared to the AS group, which was reflected in a significant difference at the 30 min time point in the OGTT graph and in a decreased OGTT area under the curve (AUC) (**Figures 1A,B**). There was no difference in insulin concentrations following OGTT in female offspring (**Figures 1C,D**). In male mice, there was no change across the OGTT or in the AUC between the groups (**Figures 1E,F**). Similarly, no significant differences were seen in the insulin response curve following the OGTT (**Figure 1G**) or in the insulin AUC in male mice between groups (**Figure 1H**).

Adipocyte Hypertrophy

Gonadal Adipose Tissue

Average adipocyte size was significantly increased in female offspring in the maternal Fr group as compared to CD group, with AS group trending higher compared to CD group ($p = 0.067$) (**Figures 2A,B**). Adipocyte distribution varied between the diets in female offspring, with AS and Fr groups reduced at 1–2,000 μm^2 in size and Fr group increased at 8–9,000 μm^2 and >10,000 μm^2 compared to CD group (**Figure 2C**).

There was an overall increase in the average adipocyte size in both the AS and Fr male offspring groups compared to CD group (**Figures 2D,E**). In male offspring, CD adipocyte distribution peaked between 1 and 4,000 μm^2, while AS and Fr distribution peaked at the larger size of 4–7,000 μm^2. AS and Fr male mice had les adipocytes at <1,000, 1–2,000, and 2–3,000 μm^2 compared to CD, but more adipocytes in the size range of 6–7,000, 7–8,000, and 9–10,000μm^2 compared to CD mice. Further, a maternal Fr diet increased the number of adipocytes at 5–6,000 and 8–9,000 μm^2 compared to CD (**Figure 2F**).

Subcutaneous Adipose Tissue

There was no overall effect following maternal AS consumption on adipocyte distribution in female offspring. However, Fr female

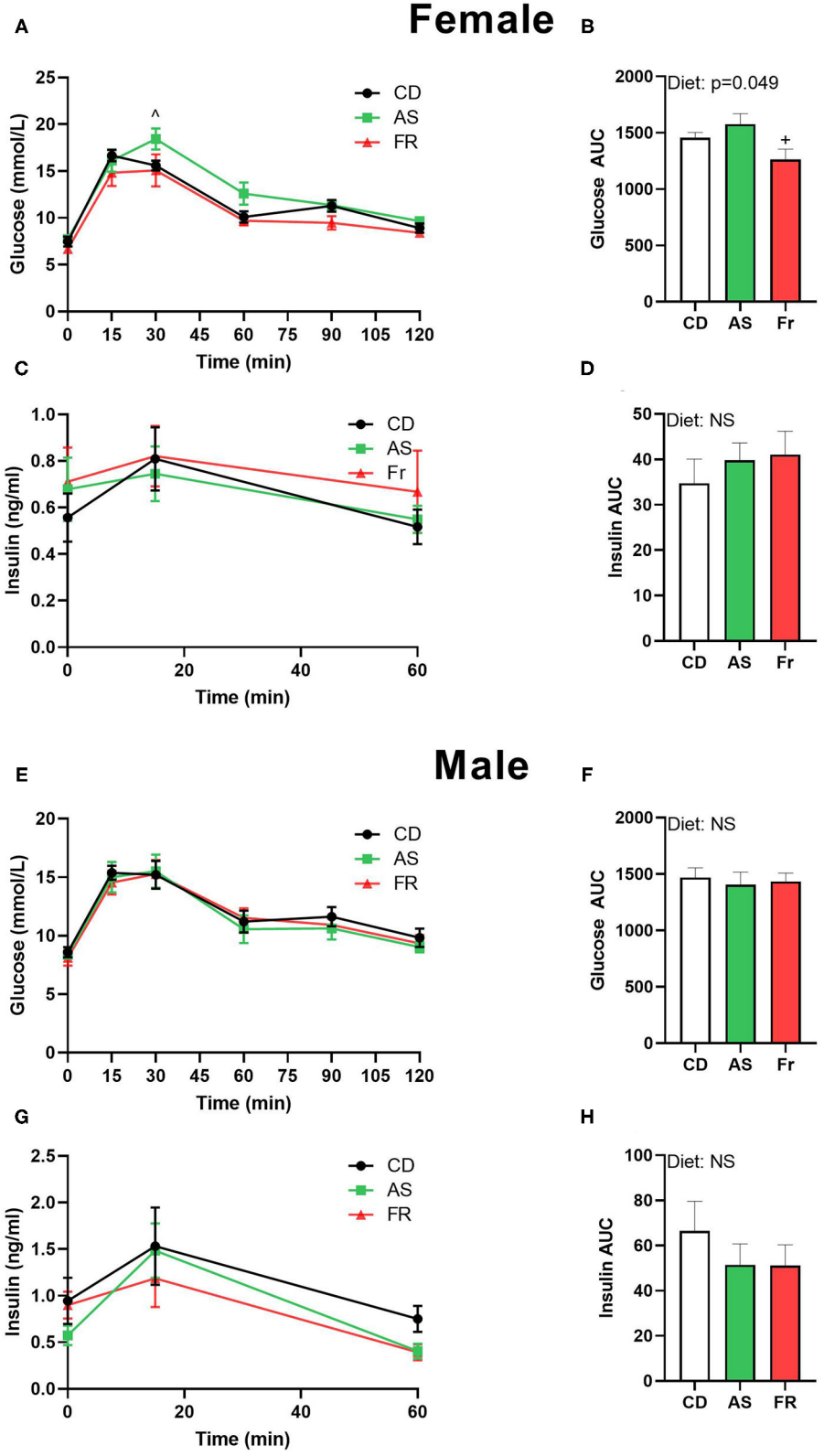

FIGURE 1 | The impact of maternal Ace-K (AS) and fructose (Fr) intake compared to Controls (CD) on glucose homeostasis at 12 weeks of age in female and male C57BL/6 offspring. OGTT (2 g/kg) in female **(A)** and male **(E)** offspring. Area under the OGTT curves in female **(B)** and male **(F)** offspring. Plasma insulin secretion curve at 0, 15, and 60 min post-OGTT in female **(C)** and male **(G)** offspring. Area under the curve in insulin in female **(D)** and male **(H)** offspring. OGTT data were analyzed using repeated measures ANOVA, AUC by ANOVA. Data are expressed as mean ± SEM. $+P < 0.05$ w.r.t AS. $\wedge P < 0.05$ w.r.t Fr; $n = 8$ per sex per group.

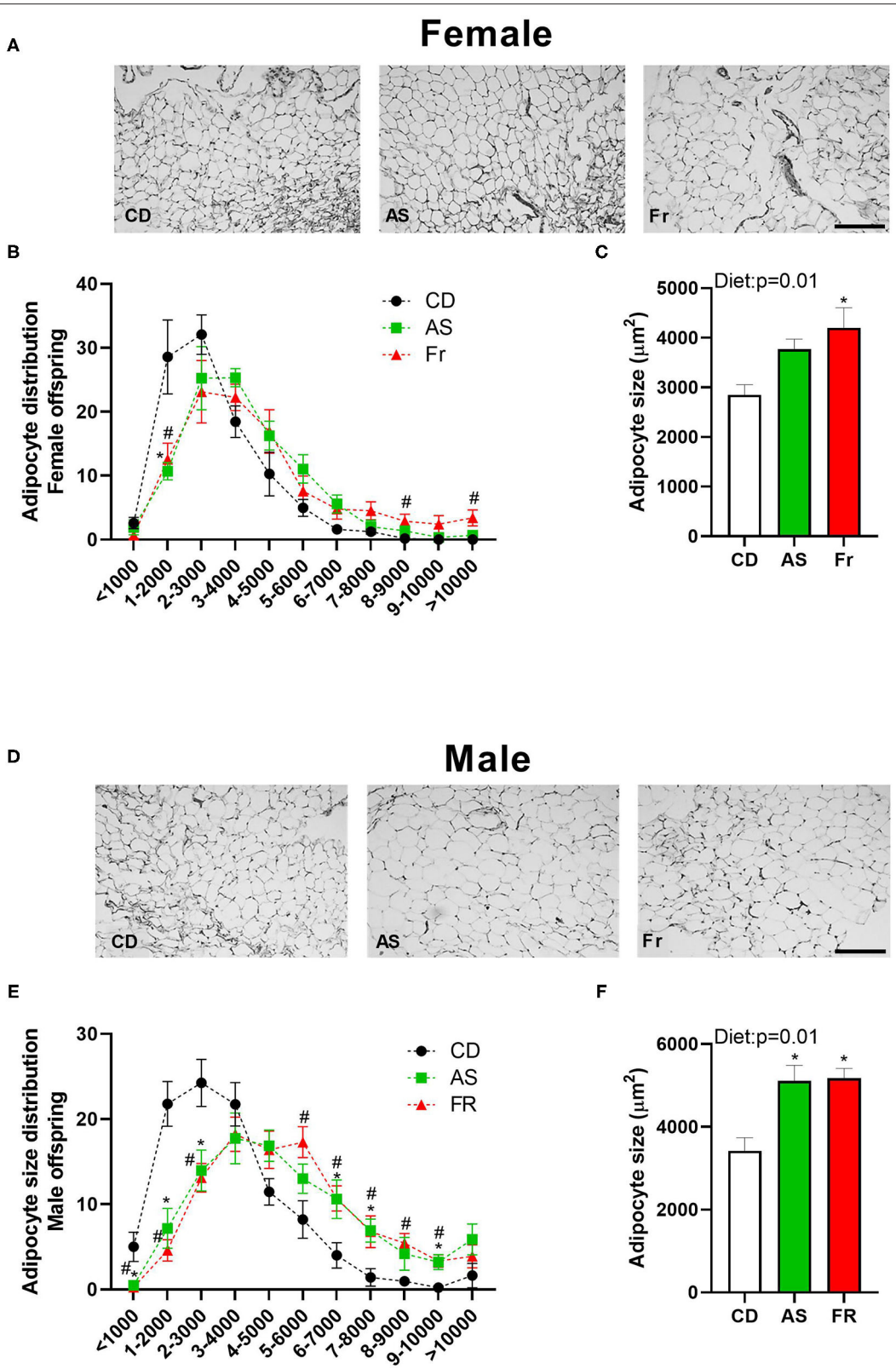

FIGURE 2 | The impact of maternal Ace-K (AS) and fructose (Fr) intake compared to Controls (CD) on adipocyte size in gonadal adipose tissue in female and male C57BL/6 offspring. Representative gonadal adipose tissue sections (hematoxylin and eosin staining) in female **(A)** and male **(D)** offspring. Adipocyte size distribution in female **(B)** and male **(E)** offspring. Adipocyte average size in female **(C)** and male **(F)** offspring. Data were analyzed by one-way ANOVA. Data are expressed as mean ± SEM. *$P < 0.05$ AS w.r.t CD. #$P < 0.05$ Fr w.r.t CD; $n = 8$ per sex per group.

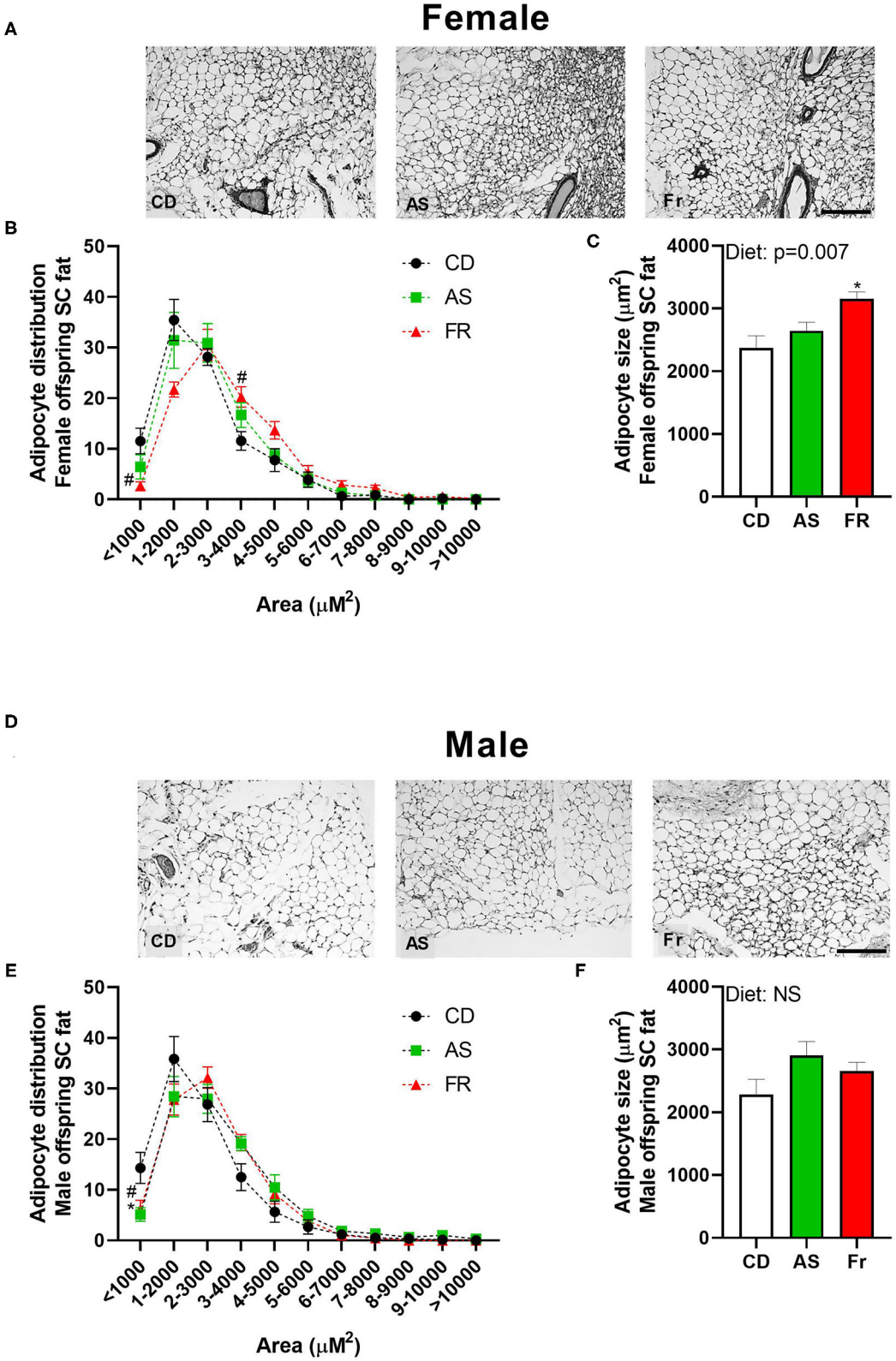

FIGURE 3 | The impact of maternal Ace-K (AS) and fructose (Fr) intake compared to Controls (CD) on adipocyte size in subcutaneous adipose tissue in female and male C57BL/6 offspring. Representative subcutaneous adipose tissue sections (hematoxylin and eosin staining) in female **(A)** and male **(D)** offspring. Adipocyte size distribution in female **(B)** and male **(E)** offspring. Adipocyte average size in female **(C)** and male **(F)** offspring. Data were analyzed by one-way ANOVA. Data are expressed as mean ± SEM. *$P < 0.05$ AS w.r.t CD. #$P < 0.05$ Fr w.r.t CD; $n = 8$ per sex per group.

offspring displayed less adipocytes at <1,000 μm^2 and more adipocytes at 3–4,000 μm^2 compared to CD (**Figures 3A,B**), with an overall increase in average adipocyte size (**Figure 3C**). In male mice, there was a decrease in the number of adipocytes <1,000 μm^2 in both the AS and Fr groups compared to the CD group (**Figures 3D,E**), although there was no significant overall difference in average adipocyte size (**Figure 3F**).

Adipose Tissue Gene Expression

There were no differences in *Fasn* expression in male or female offspring, but there was a strong trend toward an overall maternal dietary effect in females ($p = 0.052$, **Figure 4**). There were no other differences in gene expression in either male or female offspring across the different maternal dietary groups for Cd68 molecule (*Cd68*), insulin receptor substrate 1 (*Irs1*), forkhead box O1 (*Foxo1*), SRY-box transcription factor 9 (*Sox9*), tumor necrosis factor alpha (*Tnfα*), delta-like non-canonical notch ligand 1 (*Dlk1*), toll-like receptor 4 (*Tlr4*), leptin receptor (*Lepr*), nuclear factor kappa-light-chain-enhancer of activated b cells (*Nfkb*), solute carrier family 2 member 4 (*Slc2a4*), peroxisome proliferator-activated receptor gamma (*Pparγ*), peroxisome proliferator-activated receptor gamma coactivator 1 (*Ppargc1*), or interleukin-1 (*Il-1*).

Female Puberty Onset and Estrus Cycle Disruption

Maternal diet had no impact on age of puberty onset or body weight at puberty onset in females (**Table 1**). Female offspring of mothers fed with AS and Fr were more likely to experience irregular estrus cycles compared to CD groups, with those in the Fr group more likely to have persistent estrus compared to all others (**Figure 5**).

Ovarian Gene Expression

Maternal consumption of Fr reduced the expression of progesterone receptor (*Pgr*) compared to AS, inducing a significant overall dietary effect (**Figure 6A**). Cytochrome P450 Family 17 Subfamily A Member 1 (*Cyp17a1*) similarly saw Fr expression reduced compared to AS, with a significant dietary effect (**Figure 6B**). There were no differences in ovarian gene expression for follicle stimulating hormone receptor (*Fshr*), leptin receptor (*Lepr),* growth differentiation factor 9 (*Gdf9*), forkhead Box O3 (*Foxo3a*), bone morphogenetic protein 15 (*Bmp-15*), estrogen Receptor 2 (*Esr2*), luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), hydroxysteroid 17-beta dehydrogenase 1

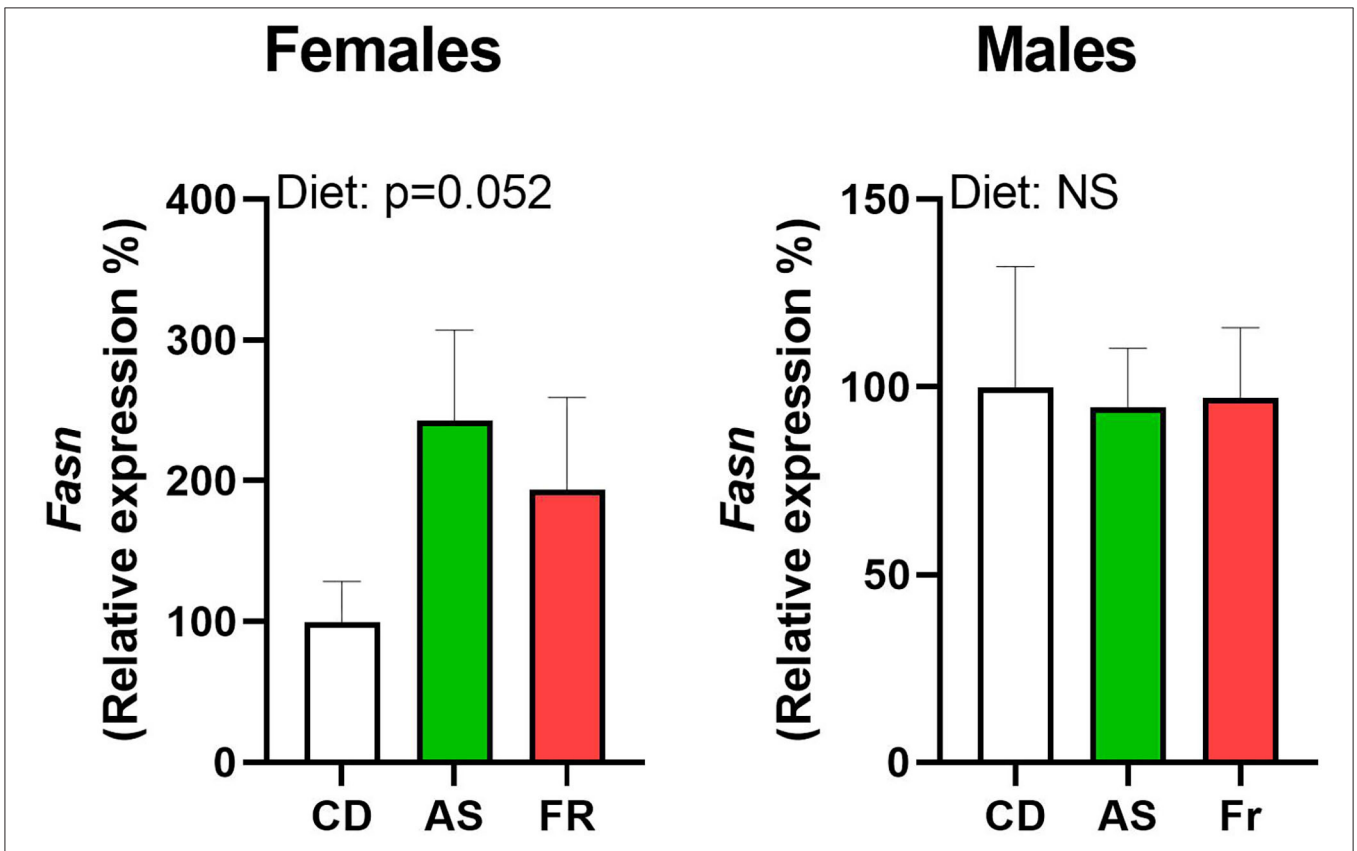

FIGURE 4 | The impact of maternal Ace-K (AS) and fructose (Fr) intake compared to Controls (CD) on *Fasn* gonadal tissue gene expression in female and male C57BL/6 offspring. Data were analyzed using one-way ANOVA. Data are expressed as mean ± SEM. n = 8 per sex per group.

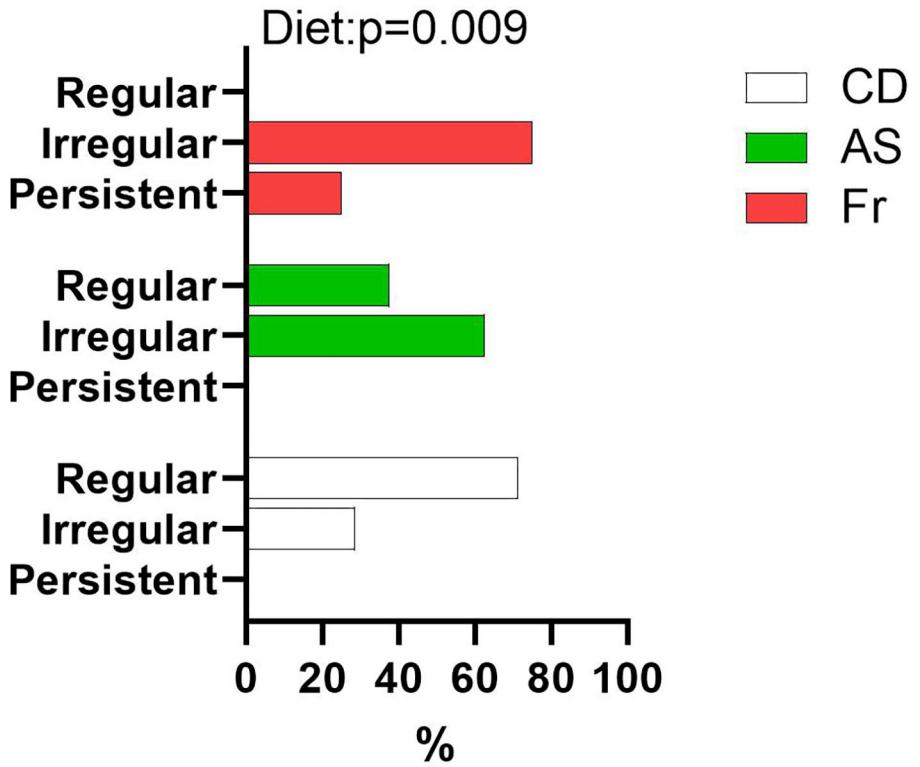

FIGURE 5 | Proportion of female offspring with regular, irregular, or persistent estrus cycles as a percentage of total female offspring within each group. Data were analyzed using nominal logistic regression. $n = 8$ per group.

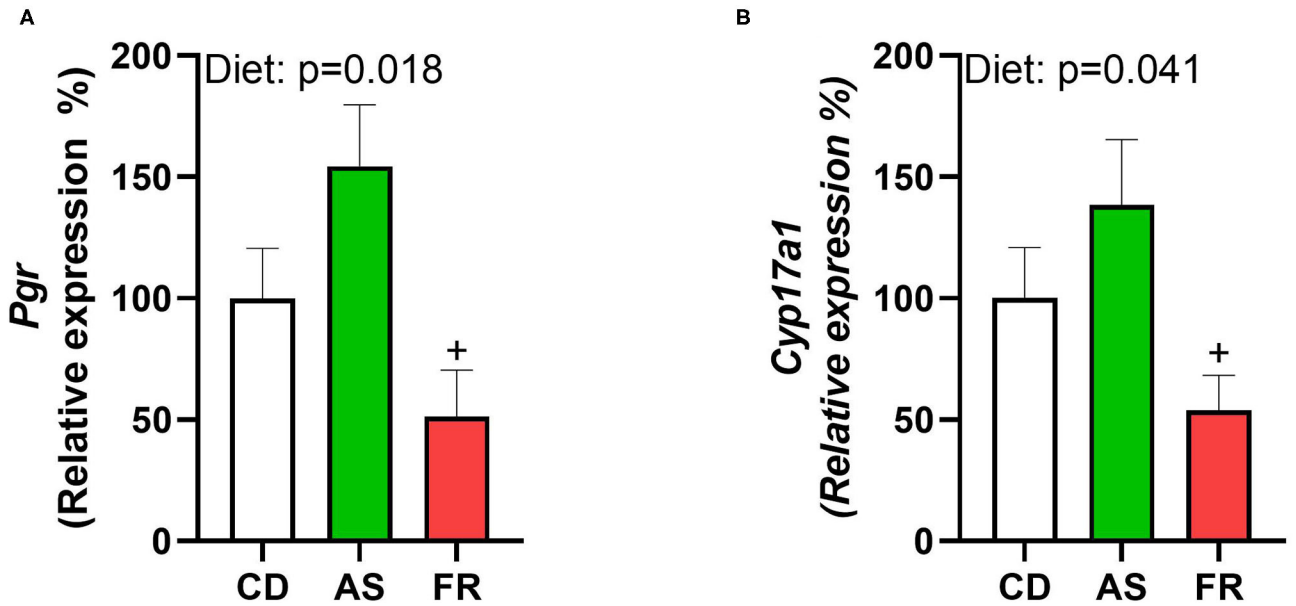

FIGURE 6 | The impact of maternal Ace-K (AS) and fructose (Fr) intake compared to Controls (CD) on ovarian gene expression in female C57BL/6 offspring. **(A)** *Pgr*, **(B)** *Cyp17a1*. Data were analyzed using one-way ANOVA. Data are expressed as mean ± SEM. +P < 0.05 w.r.t AS; $n = 8$ per group.

(*Hsd17a1*), or Epiregulin (*Ereg*) across the different maternal dietary groups.

DISCUSSION

Epidemiological, clinical, and experimental evidence have shown a clear link between an altered early-life environment and increased risk for a range of disorders in offspring in later life. In particular, maternal dietary stressors, such as sugar-sweetened soft drinks high in fructose, contribute to growing rates of metabolic disease in offspring (25). Despite their growing significance in the modern diet, the influence of AS beverages in the maternal diet is less well-known, especially regarding their impact on the metabolic and reproductive health of the offspring. The current study, therefore, utilized a mouse model to examine the effects in offspring following maternal intake of Ace-K, an AS found in most diet soft drinks (22), yet one which is less comprehensively studied. A moderate dose was chosen to better reflect the typical consumption in humans. Despite no change in weight gain, AS and Fr maternal intake increased adipocyte hypertrophy in both male and female mice and altered glucose tolerance in a sex-specific manner. Further, AS and Fr could influence female offspring reproductive systems through alteration of markers associated with ovulation, androgen conversion, and increased risk of irregular estrus cycles.

In a previous study in mice, we reported that AS in the maternal diet reduced male but not female fetal weight, while AS and Fr increased female placental weight compared to CD (15). Neither AS nor Fr maternal consumption altered offspring weight at P2, weaning, or at cull, indicating that the results of this study are independent of body weight. These results may also indicate the possibility of catch-up growth in the male offspring. Few studies have investigated the influence of maternal Ace-K consumption on the health of offspring in mice. However, other studies in mice examining maternal sucrose or sucralose intake have also reported no changes in body weight of offspring (26), while data in the rat in the setting of a maternal Fr diet also reported no differences in birth weight (27). Our results do conflict with studies in human cohorts, which have implicated maternal AS consumption in increased weight at birth and at 7 years of age (12, 14). However, these human cohorts do not distinguish between different AS and may be further influenced by confounding factors and overall dietary patterns that make it difficult to delineate the specific influence of individual sweeteners.

In male offspring, maternal AS consumption reduced plasma glucose concentrations at cull, compared to both the CD and Fr groups, although there was no impact on glucose tolerance following the OGTT. This protective effect was not seen in female offspring at cull, with the reverse being seen, whereby the OGTT revealed that glucose intolerance was increased in female offspring of mothers fed with AS compared to the Fr group. Several studies link maternal fructose diets to increased obesity and IR in offspring (28). In many studies, different concentrations of Fr or AS may cause differences in outcome, with supraphysiological concentrations likely to exacerbate the effects. Further, while Saad et al. used a similar amount of Fr in their diets (10–15% kcal/d compared to 20% kcal/d in this study), their mice were 1 year old at the time of cull (28) compared to 14 weeks in the current study. Aging is often associated with reduced efficacy of adipocytes (29), and may therefore further influence the negative effects seen in these mice. It would therefore be of interest to extend this current experiment and follow mice through to an older age to determine if obesity or other metabolic effects are exacerbated with age. Furthermore, there is evidence that maternal diet-induced developmental programming primes offspring metabolic dysfunction, and therefore this requires a "second hit" such as a post-natal high-fat diet (HFD) to demonstrate the negative impact of *in utero* exposures and reveal any latent disease (30).

With this in mind, we assessed adipocyte morphology. Despite no difference in weight gain, maternal Fr and AS induced adipocyte hypertrophy in gonadal adipose tissue in male offspring. In females, this was only seen in response to Fr, though the AS group did display a strong trend toward increased adipocyte size when compared to CD group. In both male and female offspring, the distribution of adipocytes was skewed toward larger sizes following both Fr and AS exposure. Adipocyte hypertrophy, even in the absence of obesity, is a common predictor of adipose metabolic dysfunction, dyslipidemia, IR, and T2DM. It is linked to increased recruitment of macrophages, and other pro-inflammatory adipokines (31, 32). This is especially true in visceral adipose tissue depots, which are more metabolically active than subcutaneous adipose tissue (33), though subcutaneous adipose tissue can still contribute to metabolic dysfunction when impaired (34). Hypertrophy of subcutaneous adipocytes was seen in female offspring of Fr-fed mothers, but not male offspring, indicating that female offspring might have a more systemic influence from the mother's diet on their adipose tissue morphology than male offspring.

To further investigate the influence of maternal AS and Fr intake on offspring metabolic health, a panel of genes associated with adipogenesis, inflammation, and specific metabolic pathways were examined. Female, but not male offspring, displayed a strong trend toward increased expression of *Fasn* following maternal AS and Fr intake. *Fasn* is involved in lipogenesis and associated with reduced insulin sensitivity and obesity, with expression increased by insulin within human adipocytes (35). This trend in female offspring could be indicative of an increased risk to insulin sensitivity and later-life fat accumulation (35). Despite evident adipocyte hypertrophy, male offspring exhibited no differences in the expression of genes examined in this study, indicating that male offspring may not suffer from the same negative perturbations in these investigated pathways. As previously mentioned, one theory associated with the role that maternal nutrition plays in the later health of offspring is that it primes offspring for later metabolic dysfunction that may become apparent after a secondary nutritional or environmental derangement, such as an HFD. Further, AS has been found in both amniotic fluid and breast milk (16, 17), with suggestions that this may alter the gut microbiome of the offspring and/or change their taste preference

(18). A switch toward a sweet preference could result in the offspring favoring high-sugar foods in later life, a diet associated with increased obesity, and therefore inducing this secondary nutritional challenge. In our study, offspring were maintained on water and standard control diet until cull, therefore we were unable to test whether a second insult would exacerbate any potential metabolic disease. However, if female offspring from the AS group are more susceptible to fat accumulation, later changes in diet could potentially produce these latent effects.

While the influence of maternal nutrition on the metabolic health of offspring has been frequently investigated, less is known about the potential impact of maternal diets that include AS and Fr on the reproductive health of offspring. While puberty onset was found to be unchanged between female offspring, irregular estrus cycles were increased in female offspring of AS and Fr mothers. Maternal HFD exposure has been shown to induce increased estrus cycle irregularity in female mice offspring (36), confirming the ability of altered maternal nutrition to influence aspects of reproductive health of offspring. Leptin and insulin deficiency have both been associated with alterations to female reproductive capacity; however as there was no change in either, it is likely that another mechanism is influencing the estrus cyclicity of female offspring. We assessed ovarian gene expression to further investigate estrus irregularities. *Pgr* and *Cyp17A1* gene expressions were both reduced following maternal Fr consumption compared to AS. PGR is expressed in granulosa cells of preovulatory follicles and mediates the effects of progesterone, which is involved in the regulation of ovarian function and ovulation (37). Female offspring of rat dams fed with fructose also displayed reductions in ovarian expression of *Pgr*, with maternal fructose consumption implicated in the impairment of estradiol homeostasis in female offspring (38). Cyp17A1 is involved in the conversion of progesterone to 17α-hydroxypregnenolone and then to androgens. High expression of CYP17A1 has been implicated in increased androgen concentrations commonly found in women with PCOS (37, 39), as has irregular menstrual cycles (40). Like PGR, CYP17A1 is regulated by luteinizing hormone (LH). It is possible that offspring of AS mothers have higher LH concentrations, which cause this increase in ovarian gene expression, while Fr may influence estradiol synthesis. It would be worth further exploring reproductive function and related steroid hormones within the female offspring.

Fructose increased plasma testosterone in male, but not female, offspring compared to both CD and AS groups. This agrees with a previous study where peripubertal male Wistar rats were fed Fr for 30 days, resulting in increased plasma testosterone concentrations and impaired testicular and epididymal development (41). This study was particularly interested in the reproductive health of female offspring, given the propensity to influence the next generation and the subjectivity around assessing the puberty onset of the male offspring. However, given this association between increased testosterone and modified male reproductive systems, and increasing interest in the role of paternal factors in developmental programming (42), further studies examining the influence of maternal AS and Fr intake on male reproductive health are warranted.

This study utilized a model of altered maternal nutrition in the mouse, whereby AS intake was equivalent to a standard can of diet soda a day. While many studies utilize supraphysiological concentrations of AS and Fr to induce effects, we have been able to show that lower concentrations can exert an influence on offspring following maternal consumption. Undertaking research across other ranges of AS and Fr intakes could help further elucidate the impact of diet soda consumption on the health of offspring. It would also be worthwhile undertaking a comparative study across a broader range of AS, including natural-based sweeteners such as Stevia. Further, our study ceased at 14 weeks of age. At times, the effects of developmental programming seen in offspring studies are subtle, as larger disturbances to metabolic health can increase the chance of mortality. A second insult in later life may be required to exacerbate potential disease. It would therefore be interesting to continue this study paired with a HFD in later life to examine possible interactions between maternal exposures and later susceptibility of offspring to diet-induced obesity. However, the results of the present study do indicate an impact, albeit subtle, of early life exposure to AS and Fr on later health outcomes. It may also be of interest to investigate the same parameters in the setting of maternal obesity and/or GDM, as this may exacerbate metabolic dysfunction in the offspring. This study did not investigate the gut microbiome of offspring. Given the recent insight into the influence of the gut microbiome on systemic health and the ability of AS to influence the gut microbiome, it would be a worthwhile avenue to investigate (5, 43). Further, due to limited samples, the analysis of plasma proteins and steroid hormones could not be performed. These might have given further insights into the metabolic and reproductive health of the animals. Nonetheless, PCR analysis enabled valuable analysis of a number of metabolic and reproductive markers.

In summary, a maternal diet of AS and Fr over pregnancy and lactation induced subtle metabolic and reproductive effects in male and female offspring, which were often sex-specific. Female offspring were more susceptible to glucose intolerance following a maternal AS diet. Maternal Fr and AS intake negatively impacted estrus cyclicity, and potentially impaired ovarian steroid hormone synthesis, although more investigation is required to fully elucidate these effects. Conversely, male offspring exhibited reduced baseline glucose concentrations in the setting of a maternal AS diet, and no alterations to metabolic adipose markers, thereby emphasizing the importance of undertaking studies in both males and females. Finally, the demonstrated adipocyte hypertrophy may indicate the potential for the development of later metabolic dysfunction should the offspring be exposed to another negative environmental influence, such as a postnatal HFD. As such, the present study adds to the experimental evidence to date suggesting that AS may not be beneficial alternatives to sugar-sweetened products consumed during pregnancy and early infancy.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee at the University of Auckland (Approval number 001846) in accordance with the New Zealand Animal Welfare Act, 1999.

AUTHOR CONTRIBUTIONS

Conceptualization was done by CR. Methodology was carried out by CR, PB-C, and MV. Data collection was done by CR, AS, JR, JM-J, and PB-C. Data analysis was performed by CR, PB-C, and JR. Writing—original draft preparation was done by PB-C. Writing—review and editing was done by CR, PB-C, and MV. Supervision was carried out by CR and MV. Project administration, was done by CR and MV. Funding acquisition was taken care of by CR. All authors have read and agreed to the published version of the manuscript.

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Maternal Obesity Related to High Fat Diet Induces Placenta Remodeling and Gut Microbiome Shaping That Are Responsible for Fetal Liver Lipid Dysmetabolism

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Background: Maternal obesity *in utero* may affect fetal development and cause metabolic problems during childhood and even adulthood. Diet-induced maternal obesity can impair gut barrier integrity and change the gut microbiome, which may contribute to adverse placental adaptations and increase the obesity risk in offspring. However, the mechanism through which maternal obesity causes offspring metabolic disorder must be identified.

Methods: Eight-week-old female rats received a control diet or high-fat (HF) diet for 11 weeks before conception and during gestation. The placentas were collected on gestational day 21 before offspring delivery. Placental tissues, gut microbiome, and short-chain fatty acids of dams and fetal liver tissues were studied.

Results: Maternal HF diet and obesity altered the placental structure and metabolism-related transcriptome and decreased G protein–coupled receptor 43 expression. HF diet and obesity also changed the gut microbiome composition and serum propionate level of dams. The fetal liver exhibited steatosis, enhanced oxidative stress, and increased expression of acetyl-CoA carboxylase 1 and lipoprotein lipase with changes in maternal HF diet and obesity.

Conclusions: Maternal HF diet and obesity shape gut microbiota and remodel the placenta of dams, resulting in lipid dysmetabolism of the fetal liver, which may ultimately contribute to the programming of offspring obesity.

Keywords: maternal, high-fat diet, placenta, microbiome, oxidative stress, lipid metabolism, DOHaD

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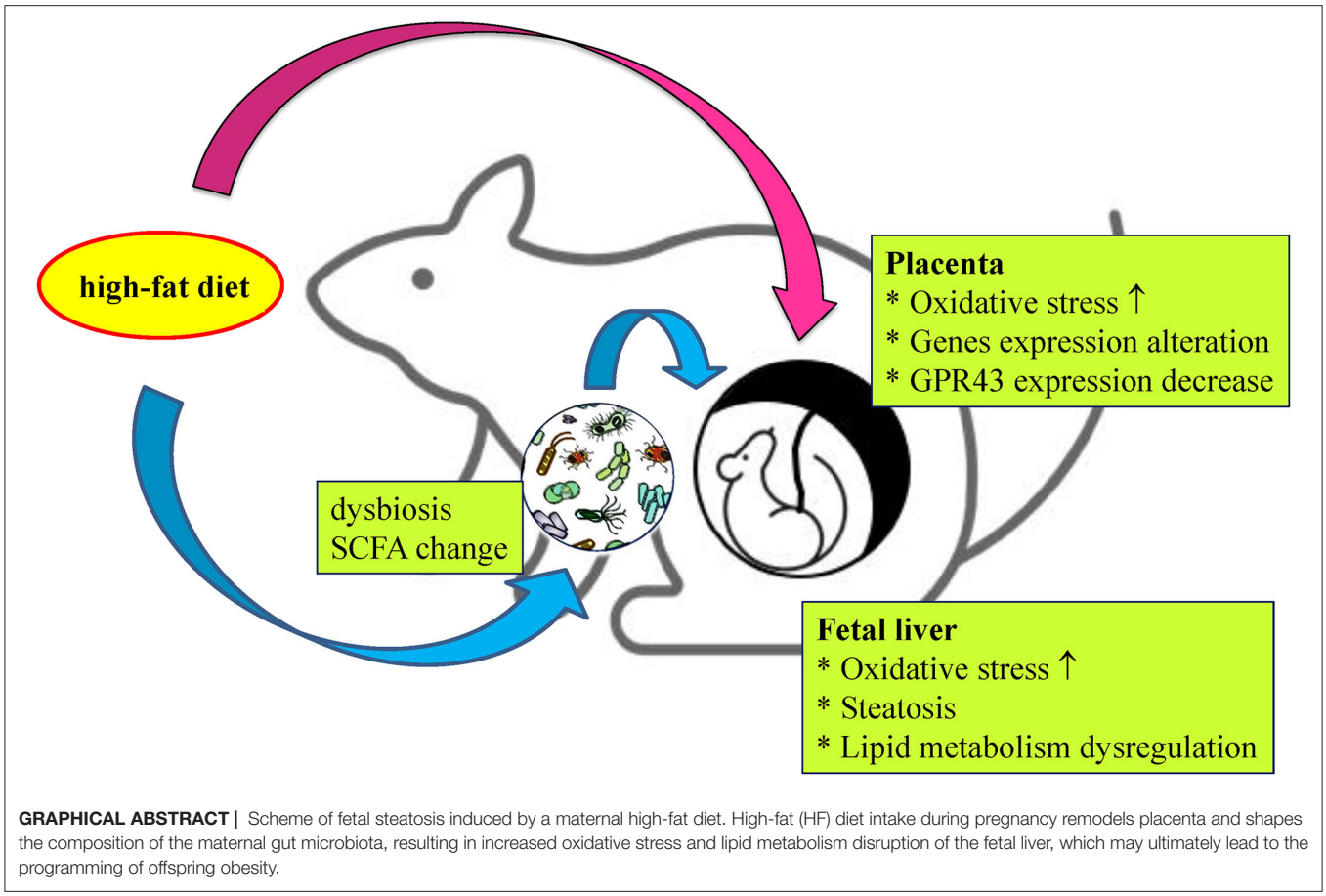

GRAPHICAL ABSTRACT | Scheme of fetal steatosis induced by a maternal high-fat diet. High-fat (HF) diet intake during pregnancy remodels placenta and shapes the composition of the maternal gut microbiota, resulting in increased oxidative stress and lipid metabolism disruption of the fetal liver, which may ultimately lead to the programming of offspring obesity.

INTRODUCTION

Obesity has rapidly increased in prevalence to become a major health problem (1). Maternal obesity can increase the risk of pregnancy complications, including pre-eclampsia, gestational diabetes mellitus, cesarean delivery, and preterm birth (2). In addition, maternal obesity and *in utero* maternal high-fat (HF) diet also affect fetal development and cause metabolic problems during childhood, adolescence, and adulthood (3). The Developmental Origins of Health and Disease (DOHaD) concept emphasizes the role of prenatal or perinatal exposure to environmental factors in determining the development of human diseases during adulthood (4). The fetus physically changes in response to environmental stress, which can increase disease risk. This programming effect is dependent on the nature and time of exposure. The DOHaD concept has been supported by numerous epidemiological and animal studies. Studies have revealed that early undernutrition impairs fetal growth and immune function in early life and increases the incidence of type-2 diabetes mellitus, cardiovascular disease, kidney disease, obesity, hypertension, osteoporosis, and metabolic syndrome later in life (4, 5). In addition to undernutrition, environmental factors such as maternal stress, infection, obesity, malnutrition, drug use, and cigarette smoke within indicated critical windows of growth and development are also associated with an increased risk of adult metabolic disease (6). Efforts to prevent non-communicable diseases have focused on adult factors; the DOHaD recommends the prioritization of optimizing nutrition early in life and reducing exposure to toxic environmental chemicals (7).

The placenta is the pivotal interface between the mother and developing embryo/fetus and plays multifunctional roles in fetal growth. Placental functions include attaching the developing fetus to the uterine wall, intervening in maternal immune tolerance, producing hormones, absorbing nutrients, removing waste, exchanging gas, and preventing the entry of chemical hazardous substances through the maternal–fetal blood supply during fetal development (8). Placental dysfunction and damage can adversely affect fetal development. The maternal nutrient supply passes to the fetus through the placenta, and the structure and function of the placenta change in response to the maternal nutrient supply. These changes affect the supply of nutrition and oxygen to and the distribution of hormones in the fetus. Evidence has indicated that obesity in pregnant women can disrupt placental function and cause adverse

pathology in the perinatal period. Structural and molecular changes in the placenta of obese dams have been reported. Kretschmer et al. reported a decreased volume fraction of the labyrinth zone with excessive lipid accumulation, impaired trophoblast differentiation, and the downregulation of cell adhesion molecules in obese maternal mice exposed to an HF diet (9). Qiao et al. reported that a maternal HF diet induces lipoprotein lipase (LPL) expression in trophoblasts of placenta accompanied by Sirtuin 1 reduction and peroxisome proliferator–activated receptor gamma (PPARγ) enhancement (10). Other mechanisms of placental damage with maternal HF diet include oxidative damage, endoplasmic reticulum stress, and changes in nutrition sensing and nutrient transport (11–14). In a previous study, we demonstrated an association between placental renin–angiotensin system (RAS) activation and HF diet–induced fetal growth restriction (15). Although we have a preliminary understanding of how maternal obesity affects the placenta, more research is required to understand how maternal obesity remodels the placenta and thus programs offspring obesity and even worsens the development of non-communicable diseases in adulthood.

A well-balanced gut microbiome is crucial for homeostasis. Intestinal microbiota are closely related to metabolic diseases, such as obesity, diabetes, and hypertension (16). Maternal HF diet changes the gut microbiome of offspring, and gut microbiome shifts are closely associated with the metabolic parameters of such offspring (17). Because the fetal gut is colonized mainly by microbiota from the vaginal and fecal microbiome of the mother during delivery, the transfer of the maternal gut microbiome may play a crucial role in offspring metabolism. Short-chain fatty acids (SCFAs), composed of less than six carbon atoms, are mainly derived through the fermentation of indigestible dietary fiber by the intestinal microbiome. SCFAs, in addition to supplying energy, can modulate metabolism and exert anti-inflammatory, antitumorigenic, and antimicrobial effects (18, 19). SCFAs are mediated through the free fatty acid receptor (FFAR). GPR41 and GPR43, also known as "FFAR3" and "FFAR2," respectively, are the most crucial receptors for SCFAs (19).

An HF diet during pregnancy and lactation has long-term consequences on the development of an offspring's gut microbiome (17). Diet-induced maternal obesity decreases the levels of maternal intestinal SCFAs and their receptors, diminishes the integrity of the gut barrier, and changes the gut microbiome, which may contribute to adverse placental adaptations and therefore increase the obesity risk in offspring (13). However, the signaling molecules that are relevant to placental adaptation and dysmetabolism in relation to maternal HF diet have not been clearly elucidated. To investigate the relationship between fetal programming and HF diet and obesity, next-generation sequencing (NGS) analysis of placentas was performed to determine the transcriptome expression after HF diet treatment. Placental adaptation and diet-induced maternal obesity change the gut microbiome and related metabolic pathways, thereby increasing the risk of obesity in offspring.

MATERIALS AND METHODS

Study Animals and Experimental Design

The experimental animal protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (approval number: 2019053001). Twelve virgin female Sprague–Dawley rats aged 7 weeks were purchased from BioLASCO (BioLASCO Taiwan, Taipei, Taiwan). The rats were housed in a light-, temperature-, and humidity-controlled environment (12-h light–dark cycle, 22°C, and 55% humidity). Food and sterile tap water were available *ad libitum* (20). After a 1-week adaption to the experimental environment (maternal age: 8 weeks), the rats were weight matched and assigned to receive either a regular control diet (59.7% carbohydrates, 27.5% protein, 12.6% fat by energy, 3.25 kcal/gm; Fwusow Industry, Taichung, Taiwan) (CC group) or an HF diet (D12331, Research Diets, New Brunswick, NJ, USA; 58% fat [hydrogenated coconut oil], 16.4% protein plus high sucrose [25% carbohydrate] by energy, 5.56 kcal/gm) (HF group) ($n = 6$ per group). The rats were fed the assigned diet for 8 weeks (maternal age: 16 weeks) and maintained in an environment conducive to mating for 3 days. The assigned diet was continued until the day of sacrifice. Mating day 1 was considered gestational day 1. The beginning of the gestation was confirmed by checking the vaginal plug. Because rats deliver their pups on day 22 or 23 of the gestation period (21), the maternal rats were sacrificed on gestational day 21 after 8 h of fasting (maternal age: 19 weeks). The offspring subjects of each group came from different litters.

Specimen Collection

The rats were sacrificed through anesthetization with a 1:1 mixture of Zoletil (25 mg/kg) (tiletamine-zolazepam, Virbac; Carros Cedex, France) and Rompun (23.32 mg xylazine hydrochloride, Bayer, Korea) administered through intramuscular injection. Heparinized blood samples were collected through cardiocentesis (22, 23). The placenta of the dams and fetal liver were collected through a cesarean section of the rats. A part of the placenta was fixed in 10% formalin in a neutral buffered solution for histological analysis, and the remainder was frozen in liquid nitrogen and stored at −80°C for NGS and quantitative polymerase chain reaction (qPCR) analysis ($n = 6$ per group). Furthermore, the retroperitoneal adipose depot was sampled through a procedure that was identical to that of our previous study (23).

Body Weight and Blood Pressure Measurement

The body weights of the rats were measured weekly from 7 weeks of age until the day of sacrifice. The blood pressure (BP) of the rats was measured at 5 days before sacrifice by using the indirect tail-cuff method (BP-2000, Visitech Systems, Apex, NC, USA) as previously described (23).

Intraperitoneal Glucose Tolerance Test

Blood sugar levels were measured using the intraperitoneal glucose tolerance test (IPGTT) after an 8-week dietary

manipulation. On the day of the IPGTT, the rats fasted for 8 h and hyperglycemia was induced through the injection of 50% glucose (2 g/kg body weight). Serum glucose levels were measured in blood from the tail vein using a glucometer (Accu-Chek, Roche, Germany) at five time points: before injection and at 15, 30, 60, and 120 min after injection. The IPGTT's integrated area under the curve (AUC) was calculated using the trapezoidal method.

Biochemical Analysis

Some plasma metabolic parameters of maternal rat blood samples were analyzed, including glutamic-oxalocetic transaminase (GOT) level, glutamic-pyruvic transaminase (GPT) level, total cholesterol (T-chol), and leptin. Serum GOT, GPT, and T-chol levels were evaluated using an automatic biochemical analyzer (Fuji Dry-chem 4400i; Fujifilm, Tokyo, Japan). Serum leptin levels were measured using an enzyme-linked immunosorbent assay kit (Abcam, Cambridge, MA, USA) ($n = 6$ per group).

Histological Analysis of the Placenta and Fetal Liver

Formalin-fixed tissues were cut into 3-μm sections by using a Leica RM2255 microtome (Leica Biosystems, Concord, ON, Canada). These sections were first stained with hematoxylin and eosin (H&E) and then scanned with a 3DHISTECH Panoramic SCAN slide scanner. The scanned image was further analyzed using Panoramic Viewer software. The placental composition was analyzed using ImageJ at 1.5× magnification. A major product of DNA oxidation is 8-hydroxy-2-deoxyguanosine (8-OHdG), which is often used as a biomarker for oxidative stress (24). The oxidative stresses of both the placenta and fetal liver were determined using 8-OhdG (25). The tissue sections were transferred to polylysine-coated slides and incubated with primary anti-8-OhdG antibody (Santa Cruz Biotechnology, CA, USA) for 60 min at room temperature. After rinsing was conducted, the sections were incubated with secondary antibody for 30 min at room temperature and thereafter incubated with Avidin and biotinylated horseradish peroxidase H. The horseradish peroxidase converted the diaminobenzidine tetrahydrochloride substrate into an insoluble dark brown precipitate. To investigate the effects of a maternal HF diet on the fatty liver and the mechanism by which this developmental priming is mediated, fetal livers were also indicated for study.

RNA Isolation, Library Preparation, and NGS and Analysis

The method for RNA isolation was identical to that described in a previous study (26). In brief, the total RNA of placental tissue was extracted using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. After quantification was conducted using an ND-1000 spectrophotometer (Nanodrop Technology, USA) and Bioanalyzer 2100 (Agilent Technology, USA), the Select Strand-Specific RNA Library Preparation Kit was used for library construction prior to the use of AMPure XP beads (Beckman Coulter, USA) for size selection. Illumina's sequencing-by-synthesis technology (Illumina, USA) was used to determine the RNA sequence. Sequencing data (FASTQ reads) were generated using Welgene Biotech's pipeline based on Illumina's base calling program bcl2fastq v2.20. After the removal of low base quality data, of polymerase chain reaction (PCR) primers, and of other artifacts, quality trimming was conducted using Trimmomatic version 0.32. Transcriptome alignment was performed using HISAT2. Reads per kilobase of exon per million mapped reads were quantified to determine gene expression. The Cuffdiff tool from the cuf?inks package was run to calculate expression changes and associated q values (P values adjusted for the false discovery rate) for each gene between the control and HF groups. Differentially expressed genes of each experiment design were subjected to an enrichment test for a functional assay by using clusterProfiler 3.5. Records in the Gene Ontology database and Kyoto Encyclopedia of Genes and Genomes (KEGG) were matched with the data using NIH DAVID Bioinformatics Resources 6.7 to determine candidate genes and pathways.

Quantitative Real-Time PCR Analysis

To validate the transcriptome expression of the placenta and evaluate the lipid metabolism of the fetal liver, the messenger RNA (mRNA) expressions of the placenta and fetal liver were analyzed through qPCR. Placentas were collected from the study rats. RNA extraction and qPCR protocols were performed per the method of previous studies (23, 27); the primer sequences of mRNA are presented in **Supplementary Table 1**. In addition, 18S ribosomal RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes for the placenta and fetal liver tissue, respectively. To evaluate the relative quantification, we adopted the comparative threshold cycle method (23, 27).

Microbial Analysis

DNA Extraction and PCR Amplification

Fecal samples for every rat were collected in individual 5-mL Eppendorf Tubes 1 week before they were sacrificed. The samples were snap-frozen with liquid nitrogen and stored at −80 °C until analysis. Microbial DNA in the stool samples was extracted using a EZNA Soil DNA Kit (Omega Bio-tek). The V3–V4 region of the bacterial 16S ribosomal RNA (rRNA) gene was amplified using PCR with primers 338F (5′-ACT CCT ACG GGA GGC AGC A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). The barcode was an N-base sequence (N represents a 6–8 nucleotide), which was unique to each sample. The PCR protocol was identical to that used in our previous study (17).

Sequencing

Amplicons were purified from 2% agarose gels by using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences). Purified amplicons were quantified using QuantiFluor-ST (Promega).

Processed amplicons were pooled in equimolar and paired-end sequences (2 × 300) and analyzed on an Illumina MiSeq platform.

Bioinformatic Analysis

The lowest sequencing reads were selected from each sample with the assistance of the pseudorandom generator, and samples were compared with respect to community composition and structure. Raw fastq files were analyzed using QIIME (version 1.17) according to the following three criteria. First, 300-bp reads were truncated at any site receiving an average quality score of b20 over a 10-bp sliding window and reads <50 bp were discarded. Second, barcodes were to be exactly matched, where primers with a nucleotide mismatch and reads containing ambiguous characters were removed. Third, only sequences overlapping for >10 bp were assembled according to their overlapped sequence; reads that could not be assembled were discarded. Operational taxonomic units were clustered with a 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed using the RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU115) 16S rRNA database at a confidence threshold of 70%. To determine whether HF diet exposure creates a similar gut microbiota pattern, we evaluated the Firmicutes to Bacteroidetes (F/B) ratio.

SCFA Analysis

To demonstrate the influence of the gut microbiota on a host, serum SCFA levels, the main fermentation product of the gut microbiota, were compared between the rats receiving an HF diet and those receiving the control diet. The plasma levels of acetic acid, propionic acid, and butyric acid were determined using gas chromatography (GC). Our previous study detailed the GC protocol (17). In brief, the 100-μL sample was mixed with 5 μL of 100-μM internal standard and 100 μL of propyl formate. After vertexing and centrifuging, the supernatant was injected for GC analysis (Shimazu QPlus 2010 gas chromatography with flame ionization detector [FID]). The injection volume was 2 μL, and the inlet and FID temperatures were 200 and 240°C, respectively.

Statistics

Differences between the HF and CC groups were analyzed through the Mann–Whitney U test for dependent variables that are not normally distributed. Values are expressed as the mean ± standard error of the mean, and a P value of <0.05 was considered statistically significant. A repeated-measure analysis of variance model was used to determine the body weight (BW) difference and for the IPGTT test between the groups. The BW of the rats was measured weekly from 8 weeks of age until sacrifice. The IPGTT was performed 5 days before sacrifice. The interaction between group and time (G × T) was calculated for each variable. All statistical analyses were performed using SPSS 22.0 for Windows XP (SPSS, Chicago, IL, USA).

RESULTS

HF Diet Causes Obesity and Alters the Metabolic Profile of Dams

The BWs of the rats were measured weekly from 8 weeks of age until the day of sacrifice (**Figure 1**). The HF group had a significantly higher BW than the CC group did after 1 week of exposure to the assigned diets (HF vs. CC; 232.42 ± 8.83 g vs. 211.83 ± 3.68 g; $P = 0.037$) until the end of the experiment (HF vs. CC; 397.06 ± 14.83 g vs. 316.13 ± 4.99 g; $P = 0.004$). Weight increased significantly in the HF group after 11 weeks of diet manipulation. Repeated measures indicated a main effect for time ($F_{11, 110} = 155.635$, $P < 0.001$) and group ($F_{1, 10} = 15.837$, $P = 0.003$) (G × T, $P < 0.001$). After 11 weeks of diet manipulation, the metabolic profiles of dams receiving an HF diet exhibited a significantly higher level of systolic BP, a larger volume of retroperitoneal fat, and higher serum leptin levels than those of dams receiving the control diet (**Figure 2**). GOT, GPT and T-chol values were similar between the CC and HF groups (**Figures 2E,F**). For IPGTT, the HF group exhibited a non-signficantly higher glucose level at 15 and 30 min than did the CC group (**Figure 2G**). The AUC was also slightly but non-significantly higher (the main effect of time [$F_{4, 40} = 31.584$, P < 0.001] and group [$F_{1, 10} = 1.427$, $P = 0.260$]; G × T, $P < 0.001$) (**Figure 2H**).

Exposure to HF Diet Changes Placental Structure and Expression of Metabolism-Related Genes

Exposure to HF Diet Decreases the Thickness of the Placental Labyrinth Zone and Increases 8-OHdG Expression

The placenta plays an essential role in fetal programming; therefore, the remodeling of the placenta by a maternal HF diet was studied. The CC and HF groups did not signifiacntly differ with respect to placental weight and litter characteristics, including number, size, and sex (**Supplementary Table 2**). Considering that the placenta contributes to direct nutrient exchange between fetal and maternal circulation, we analyzed placental adaptation due to the HF diet. The mature placenta is composed of three histologic zones, namely the maternal decidua on the outside, the junctional zone, and the inner labyrinth zone. A comparison of histologic zone thickness revealed that the labyrinth zone was significantly thinner in the HF group than in the CC group (HF vs. CC; 82.64% ± 1.26% vs. 86.25% ± 1.01%, $P = 0.038$, **Figure 3A**). Oxidative stress between the two groups was compared based on 8-OHdG staining. All three placental zones in the HF group exhibited greater 8-OHdG staining by an average of 1.67 ± 0.23 fold relative to the CC group ($P = 0.015$; **Figure 3B**).

Maternal HF Diet Changes the Expression of Metabolism-Related Genes in Placenta

An NGS analysis of placentas was performed to determine the transcriptome expression after an HF diet treatment. The total number of reads in the HF and CC groups were 84,978,988 and 100,611,252, with a mapping rate of 95.79 and 95.25%,

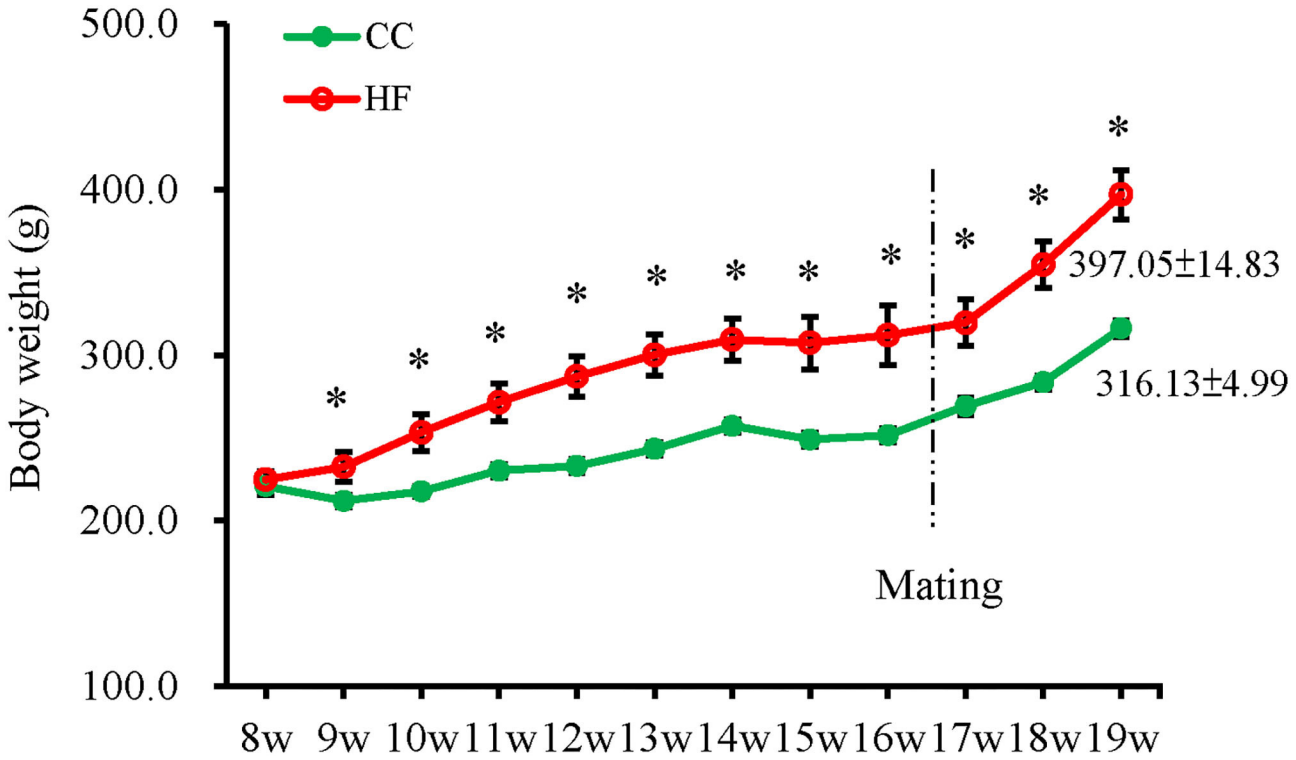

FIGURE 1 | Change in body weights of dams after control diet (CC) or high-fat diet (HF). The results are presented as the mean ± standard error. A significant difference was observed after undergoing the diet for 1 week. A repeated-measure ANOVA model was used to test the difference in BW between the groups ($n = 6$ for each group). *$P < 0.05$.

respectively. The heat map revealed a substantial difference between the HF and CC groups in terms of mRNA expression (**Figure 4A**). Those mRNA with at least a 1.5-fold difference ($P < 0.01$) in expression between the placental tissues of the CC and HF groups were selected. The log2-fold change was used to classify the genes into upregulated and downregulated groups. We observed 26 and 27 upregulated (**Supplementary Table 3**) and downregulated (**Supplementary Table 4**) genes, respectively, in the HF group compared with in the CC group. Genes with the largest difference between the two groups are illustrated in **Figure 4B**. The 10 genes with the highest expression in the HF group compared with the CC group are indicated in red; they are Srm, GSTM3, Usp9y, Spock3, Lfng, Rrad, Prl2b1, Orm1, Hmgn5b, and Smoc1. The 10 genes with the lowest expression in the HF group compared with the CC group are shown in green; they are SPARC, Ndst1, Rpl30, Prl7b1, AfP, Map2k3, Pdia5, Mff, Cpb2, Egf23, and Elovl6. Among them, five upregulated and downregulated genes each were selected for qPCR validation. The results of the qPCR were compatible with those of the NGS (**Figure 5**). DAVID v6.7 was then used to identify functionally related gene groups. Functional annotation clustering revealed 19 significantly related KEGG pathways in the placental tissues of the HF group vs. the control group (**Table 1**). Ribosome was the most significant KEGG pathway. Other functional pathways altered by the maternal HF diet include cholesterol, arginine, and proline metabolism, oxidative phosphorylation, non-alcoholic fatty liver disease, and tight junction.

HF Diet Shapes the Gut Microbiota and Decreases the Plasma Propionate Level of Dams

To illustrate how maternal HF diet exposure changes the gut microbiome and then influences the metabolic parameters of the offspring, we determined the proportions of 16S rDNA reads assigned to each phylum for dams. The HF group exhibited a lower level of alpha diversity than did the CC group in terms of the Shannon index (**Figure 6A**). The beta diversity determined through a principal coordinate analysis indicated no significant difference between both groups (data not shown). The CC and HF groups exhibited distinct patterns both at the phylum (**Figure 6B**) and genus (**Figure 6C**) levels.

Although the F/B ratio increased in the HF group in our study, this increase was not statistically significant. Subsequently, the taxa abundance of the HF and CC dams was determined through linear discriminant analysis (LDA) and effect size (LEfSe) analysis (**Figure 7**). LEfSe analysis is performed to identify differences in flora and types of microorganisms between groups, which aids the development of biomarkers (17). The size of the

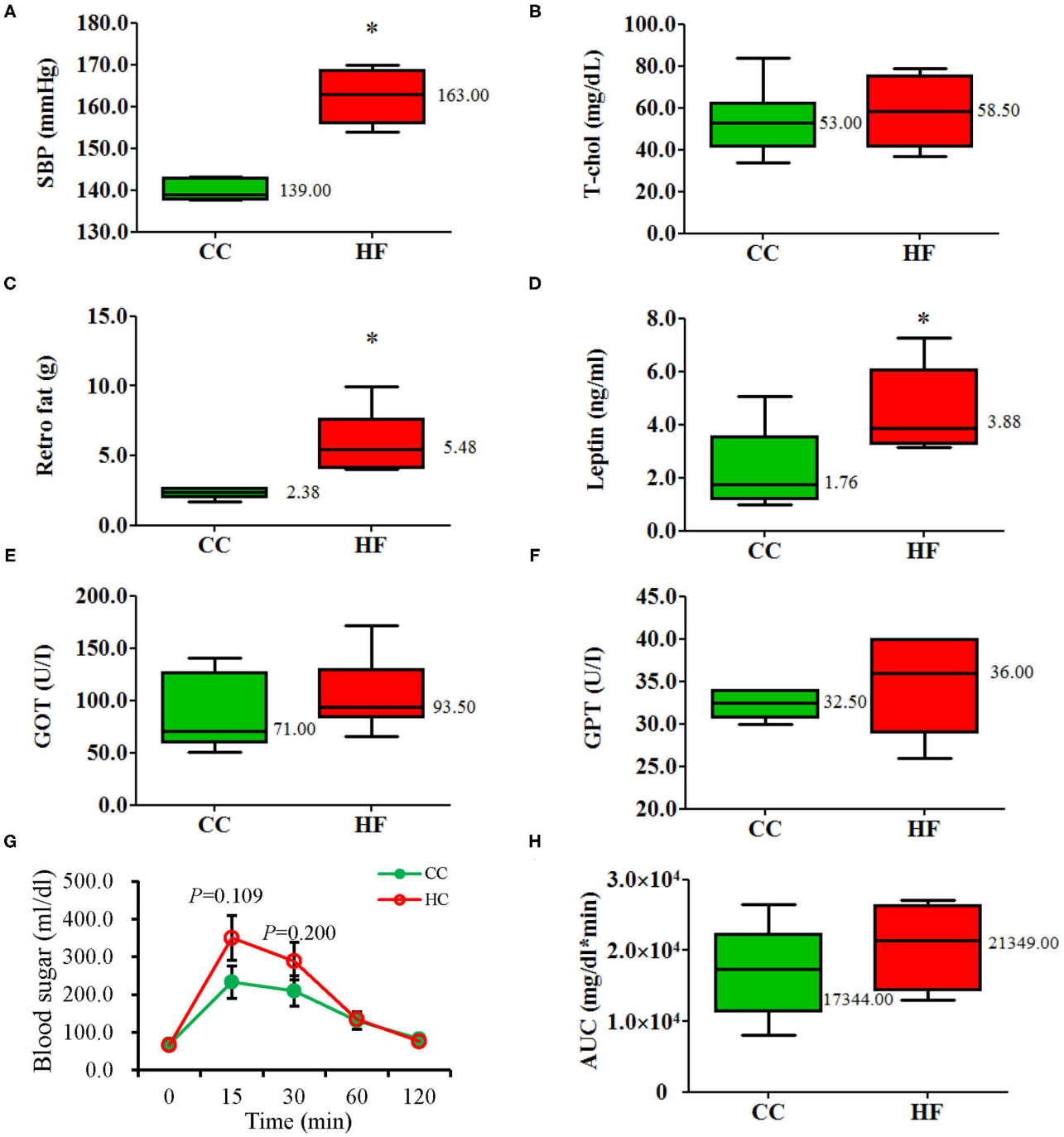

FIGURE 2 | Metabolic profiles of the dams after 11 weeks of undergoing the diet (19-week-old). **(A)** SBP, **(B)** T-cho, **(C)** Retro fat, **(D)** Leptin, **(E)** GOT, **(F)** GPT, **(G)** blood sugar, **(H)** AUC. Compared with the control group (CC), the high-fat diet group (HF) had higher systolic blood pressure, greater retroperitoneal fat deposits, and higher plasma leptin levels. The integrated AUC values of the intraperitoneal glucose tolerance test were calculated using the trapezoidal method. All values are presented as mean ± standard error. A repeated-measure ANOVA model was used for the IPGTT test of the groups. Other parameters were analyzed using a Mann–Whitney *U* test ($n = 6$ for each group). *$P < 0.05$. The median was shown. SBP, systolic blood pressure; Retro, retroperitoneal, GOT, glutamic-oxalocetic transaminase; GPT, glutamic-pyruvic transaminase; T-chol, total cholesterol; AUC, glucose area under the curve.

different species is represented by the length of the histogram (i.e., LDA score) (28). The histogram revealed that HF dams (red) had more of the *Romboutsia* genus and *Akkermansia* genus than did the CC dams (green). However, the CC dams had more of the *Lachospiraceae* genus than did the HF dams. The genera that were increased in the HF group belonged to the Firmicutes phylum (**Figure 7C**). The predicted functions of different gut microbiomes between CC and HF groups indicated

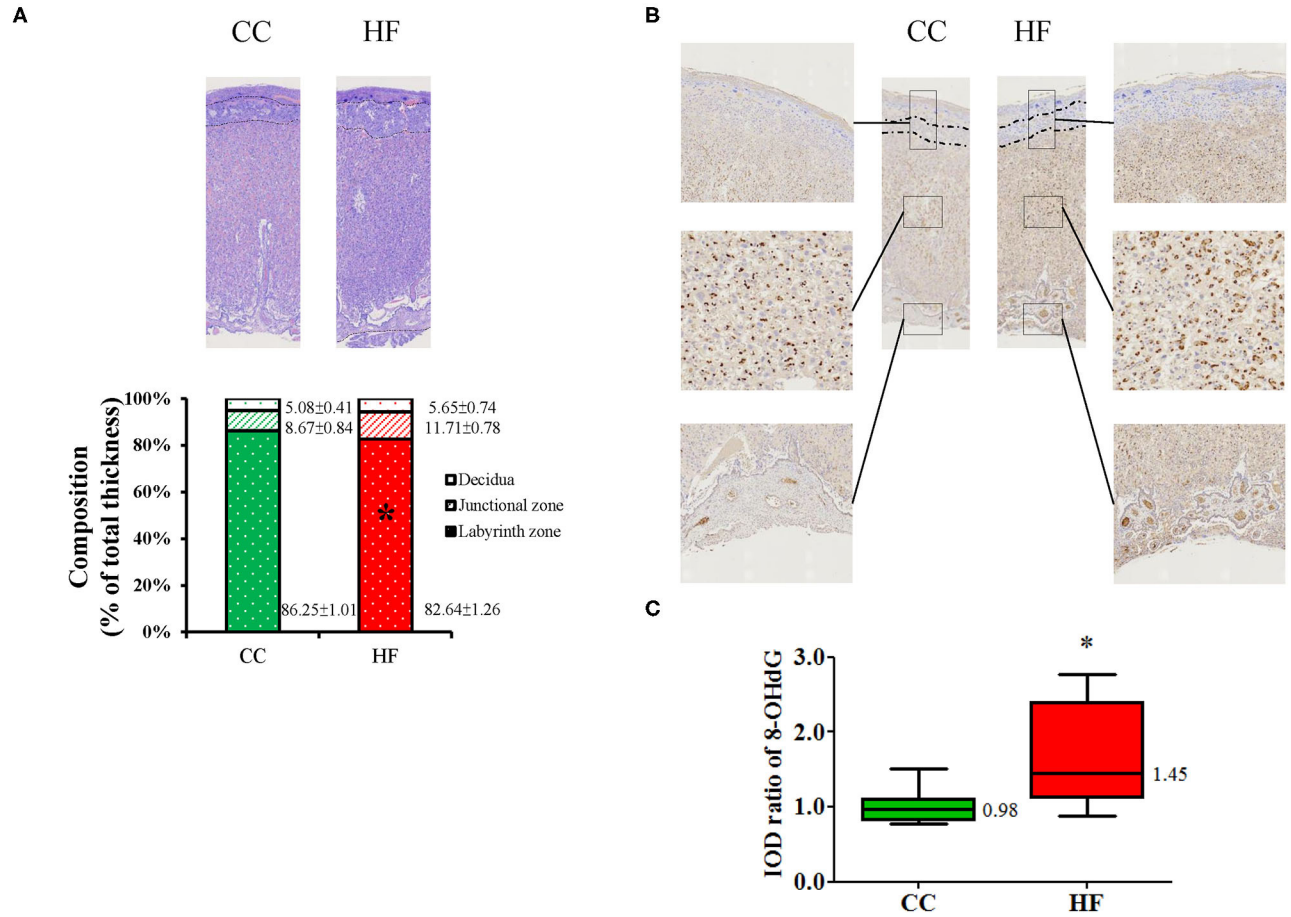

FIGURE 3 | Histological changes in placenta after high-fat diet intake. **(A)** Histological appearance of placenta. Mean proportions of thicknesses of the decidua basalis, junctional zone, and labyrinth zone are indicated on a bar graph. **(B)** Oxidative stress was determined based on 8-hydroxy-2-deoxyguanosine (8-OHdG). **(C)** Relative quantitative analysis of 8-OHdG by IOD. Magnification of boxed area detailing the three layers of placenta and chorionic villi (V) ($n = 6$ for each group). *$P <$ 0.05. The median was shown. CC, control group; HF, high-fat diet group; IOD, image optical density.

a relationship with amino acids, glucose, and lipid metabolism (**Supplementary Figure 1**).

HF Diet Intake Changes Plasma SCFAs and the Gene Expression of G-Protein-Coupled Receptor 43 in Placenta

Considering that dysbiosis is associated with an HF diet, we hypothesized that SCFAs would be affected in the HF group. The plasma propionate level was significantly lower in the HF group than in the CC group; however, the levels of acetate and butyrate were similar in the two groups (**Figure 8A**). In addition to the decreased plasma propionate level in the HF group, we found that the corresponding mRNA expression of G-protein-coupled receptor (GPR) 43, an SCFA receptor, in the placenta decreased due to a maternal HF diet, whereas the mRNA expression levels of GPR41 and OLFR59 were not affected (**Figure 8B**).

Maternal HF Diet Programs Fetal Liver Steatosis Through Alteration of the key Enzyme in Lipid Metabolism

Increasing evidence indicates that non-alcoholic fatty liver disease may begin at birth or even *in utero* and may continue on to adulthood (29). The pathway of non-alcoholic fatty liver disease (NAFLD) in placental transcriptome changed significantly due to obesity and a maternal HF diet (**Table 1**). To investigate the manifestation of fetal fatty liver with maternal HF diet and the mechanism through which this developmental priming is mediated, fetal livers were sampled for further study. In a histological examination, fetal livers exhibited extensive fat deposition in the HF group relative to the CC group; this was indicated by an increased proportion of vacuolation in H&E staining (**Figure 9A**). Furthermore, the oxidative stress determined by 8-OHdG increased in the fetal livers when mothers were fed an HF diet (**Figure 9B**). To uncover the programming mechanism, we analyzed the fetal liver mRNA expression of key enzymes corresponding to lipid metabolism.

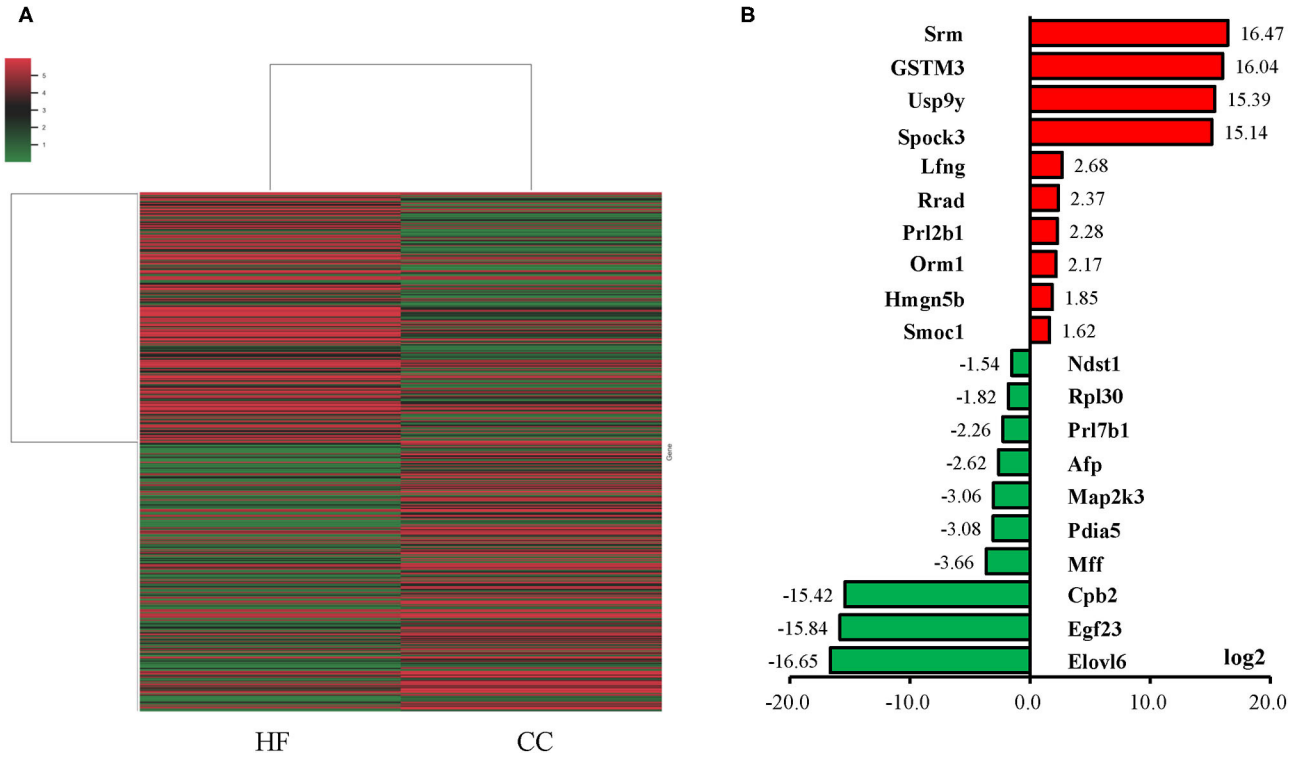

FIGURE 4 | Gene expression of placental tissue in control diet (CC) group and high-fat diet (HF) group. **(A)** Heat map of mRNA expression determined using next-generation sequencing (NGS). The heat map lane represents the top 100 expressed mRNAs. Higher and lower intensities are indicated in red and green, respectively. Among the gene expressions with a 1.5-fold difference, the top 10 mRNAs expressed in the HF (red bar) and CC groups are indicated on the bar chart **(B)**. Expression of mRNAs is presented in terms of relative folds. Srm, spermidine synthase; GSTM3, glutathione S-transferase mu 3; Usp9y, ubiquitin specific peptidase 9, Y-linked; Spock3, SPARC/osteonectin, cwcv and kazal like domains proteoglycan 3; Lfng, LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase; Rrad, RRAD, Ras-related glycolysis inhibitor and calcium channel regulator; Prl2b1, prolactin family 2, subfamily b, member 1; Orm1, orosomucoid 1; Hmgn5b, high mobility group nucleosome binding domain 5B; Smoc1, SPARC related modular calcium binding 1; Ndst1, N-deacetylase and N-sulfotransferase 1; Rpl30, ribosomal protein L30; Prl7b1, prolactin family 7, subfamily b, member 1; AfP, alpha-fetoprotein; Map2k3, mitogen activated protein kinase kinase 3; Pdia5, protein disulfide isomerase family A, member 5; Mff, mitochondrial fission factor; Cpb2, carboxypeptidase B2; Egf23, fibroblast growth factor 23; Elovl6, ELOVL fatty acid elongase 6.

The genes for coding the key enzyme involved in lipid metabolism, such as acetyl-CoA carboxylase (ACC) 1 and LPL, were significantly affected by maternal HF diet (**Figure 10**).

DISCUSSION

According to previous studies, pregnant women who are obese or who have an HF diet are more likely to have offspring with obesity or metabolic problems (20, 30–32). To explore the fetal programming mechanism, placenta adaptation and changes in the maternal gut microbiome were examined in this study. We found that maternal HF diet/obesity can lead to placental remodeling through labyrinth zone hypoplasia, oxidative stress increases, GPR43 expression decreases, and alterations in metabolism-related transcriptomes. Furthermore, a maternal HF diet altered the maternal gut microbiome and decreased serum propionate. Placental remodeling and maternal dysbiosis elicit imbalances in key enzymes in lipid metabolism, oxidative stress, and steatosis in the fetal liver (graphic abstract).

The placenta is plastic and can adapt to the maternal environment to optimize the growth and development of the fetus. Studies have demonstrated that the total cortisol level of pregnant women with obesity during pregnancy is lower than that of pregnant women (33). In addition, the placental 11β-HSD-2 barrier of obese pregnant women is also upregulated, which further reduces the glucocorticoid exposure of the fetus of obese pregnant women without obesity (34). However, the effect of obesity on the metabolism of placental glucocorticoids has not been well determined. Placental dysfunction and damage are closely related to embryonic and fetal damage (8). The labyrinth zone is the layer nearest to the fetus and is composed of maternal sinusoids, trophoblastic septa, and fetal capillaries. The proportion of the placenta constituted by the labyrinth zone increases with gestational time (8). The labyrinth zone plays a role in gaseous exchange, nutrient provision, and waste removal for the fetus. Syncytiotrophoblasts in the labyrinth zone constitute a barrier that separates fetal circulation from maternal circulation. These cells have several outflow and inflow transporters that regulate chemical transfer between the mother and fetus (8).

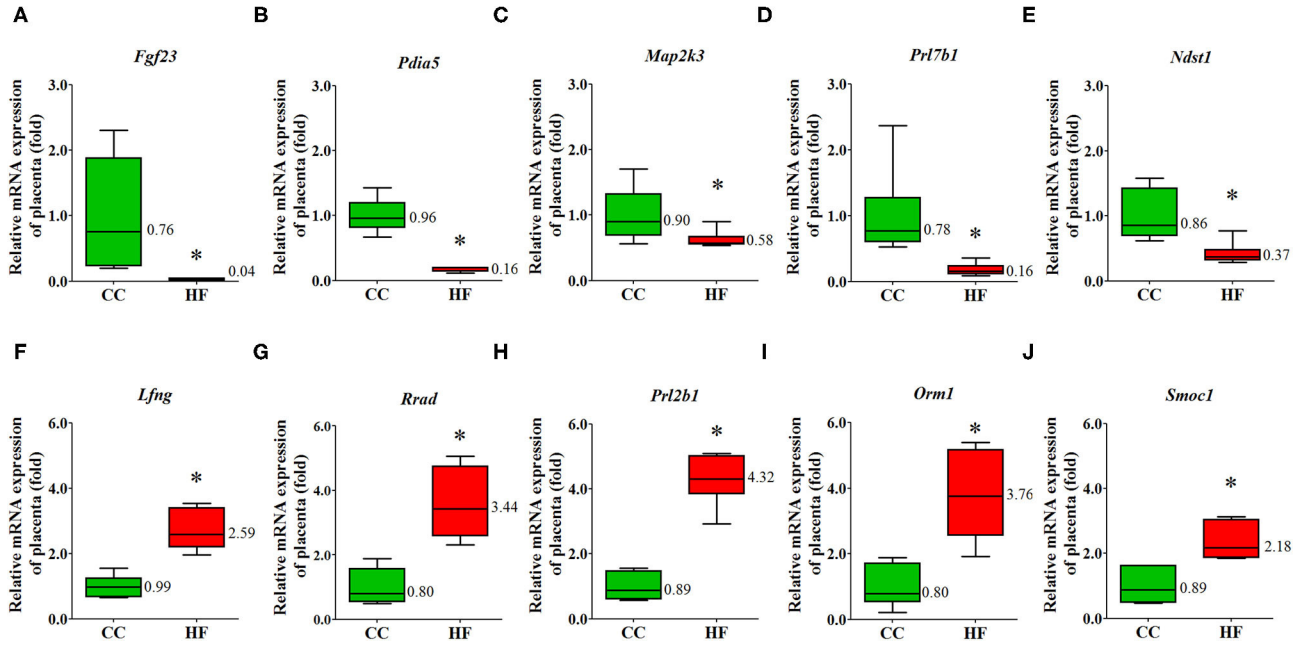

FIGURE 5 | Validation of dysregulated placental mRNA. The expression of dysregulated genes was compared between rats exposed to a high-fat diet or a control diet. Reverse transcription–quantitative polymerase chain reaction analysis was conducted to validate the mRNA profiles of five mRNAs determined using next-generation sequencing (NGS). **(A)** *Fgf23*, **(B)** *Pdia5*, **(C)** *Map2k3*, **(D)** *Prl7b1* **(E)** *Ndst1*, **(F)** *Lfng*, **(G)** *Rrad*, **(H)** *Prl2b1*, **(I)** *Orm1*, **(J)** *Smoc1*. The results demonstrated that the expression patterns of these mRNAs are consistent with those determined by NGS. The housekeeping gene was 18S ribosomal RNA, and mRNA expression is presented in terms of relative fold. $^*P < 0.05$ ($n = 6$ for each group). The median was shown. Fgf23, fibroblast growth factor 23; Pdia5, protein disulfide isomerase family A member 5; Map2k3, mitogen activated protein kinase 3; Prl7b1, prolactin-7B1; Ndst1, N-deacetylase and N-sulfotransferase 1; Lfng, LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase; Rrad, Ras-related glycolysis inhibitor and calcium channel regulator; Prl2b1, prolactin family 2, subfamily b, member 1; Orm1, orosomucoid 1; Smoc1, SPARC-related modular calcium binding 1.

Compared with other regions of the placenta, the labyrinth zone is more susceptible to toxicological damage because of its high blood flow, active cell proliferation, and long proliferation period (8). Labyrinth zone damage is related to intrauterine growth restriction (35). The labyrinth zone is a hormone-dependent tissue, and estrogen is an inhibitor of placental growth. Estrogen overproduction or drugs with estrogenic effects may induce labyrinth zone hypotrophy (36, 37). Furthermore, labyrinth zone hypotrophy can be caused by other factors, such as the presence of immunosuppressants (e.g., glucocorticoid, azathioprine, and cisplatin) or maternal undernutrition (8). Both a maternal HF diet and undernutrition can influence the labyrinth zone; thus, the labyrinth zone tissue of the placenta is also susceptible to nutrition-related effects.

Differences in the expression of some placental genes between the CC and HF groups were identified through NGS and validated through reverse transcription PCR. Spermidine can induce autophagy and exhibits antiaging effects in multicellular organisms, including nematodes, flies, and mice. Spermidine provides several beneficial effects with caloric restriction, which partially protects against cardiovascular problems and cancers in rodent models (38). Spermidine synthetase (SRM) catalyzes spermidine production from putrescine and decarboxylated S-adenosylmethionine. High-nutrient diets upregulate the gene expression of SRM in the placenta and fetal liver of mini-pigs (39). Therefore, increased SRM in the placenta of dams with HF diet exposure is a compensatory effect that must be further investigated. The mu class of glutathione S-transferase functions to detoxicate electrophilic compounds, including some carcinogens, environmental toxins, and products of oxidative stress, through conjugation with glutathione (40). Elongation of long-chain fatty acid family member 6 (Elovl6) is an enzyme that functions in the elongation of saturated and monounsaturated fatty acids with 12, 14, and 16 carbon atoms. Elovl6 was reported to play a crucial role in lipid metabolism and insulin sensitivity. Polyunsaturated fatty acids in the diet can suppress Elovl6 expression (41). Mice with targeted disruption in the gene for Elovl6 (Elovl6 −/−) are resistant to diet-induced insulin resistance (42). Huang et al. demonstrated that Actg2, Cnfn, Muc16, and Serpina3k are at the gene network core in the placental tissue and that the genes Tkt, Acss2, and Elovl6 served as the network core in the gonadal fat tissue for mice with an HF diet (43). In our study, Elovl6 gene expression was greatly repressed in the placenta of the rats with an HF diet. Thus, Elovl6 may play a crucial role in fetus priming with a maternal HF diet. Other altered KEGG pathways in the placenta transcriptomes under a maternal HF diet/obesity include oxidative phosphorylation, arginine and proline metabolism, and NAFLD. The functional pathways indicated by the gut microbiome of the dams under an HF diet and obesity are fatty acid metabolism, fatty acid elongation in the mitochondria, arginine and proline metabolism, and biosynthesis

TABLE 1 | KEGG pathways relevant to differently expressed genes in placental tissues of HF and CC groups.

Description	Gene ratio	Bg ratio	*P* value	q value	Gene ID	Count
Ribosome	28/296	179/8567	0.000	0.000	LOC108352650/Rpl26/Rps27a/Rpl19/Rpl30/Rps24/Rpl29/LOC100360522/Rps2/Rpl35/Rps12/Uba52/Rps11/Rpl27/LOC687780/Rpl23a/Rps15/Rps18/LOC102555453/LOC100362684/LOC100362027/LOC100360841/Rps19/LOC103694404/LOC100359687/Rpl7a/Rps27l/Rps10l1/	28
Complement and coagulation cascades	11/296	83/8567	0.000	0.015	Serpinc1/Fgb/Vtn/Cpb2/F2/Plat/Fga/Fgg/Serpina1/C3/C7/	11
Cholesterol metabolism	7/296	51/8567	0.002	0.072	Apoh/Apob/Apoc2/Apoa1/Soat2/Apoa4/Lipc/	7
Transcriptional misregulation in cancer	15/296	184/8567	0.002	0.072	Cdkn1a/Lyl1/Nr4a3/Slc45a3/Ewsr1/Gadd45g/Hhex/Csf1r/Plat/Pax8/LOC102549173/Hist3h3/LOC103694865/Cebpb/Igfbp3/	15
Thermogenesis	18/296	243/8567	0.002	0.072	Prkg2/Cox4i2/Ndufa6/LOC100911615/Adcy4/Ndufa13/Cox8a/Atp5mf/LOC100363268/Bmp8a/COX2/Ndufa1/Ndufa11/Adcy9/LOC100361457/Cox7b/Uqcr10/LOC103694876/	18
Arginine and proline metabolism	7/296	52/8567	0.002	0.072	Gatm/Aldh2/Maoa/Nos3/Srm/LOC100912604/Nos2/	7
African trypanosomiasis	6/296	39/8567	0.002	0.072	Il12a/Hba1/Apoa1/Hba-a1/LOC103694857/Hbb/	6
p53 signaling pathway	8/296	74/8567	0.004	0.106	Sesn1/Cdkn1a/Zmat3/Gadd45g/Pmaip1/Ccnd1/Ccnb1/Igfbp3/	8
Oxidative phosphorylation	12/296	143/8567	0.004	0.106	Cox4i2/Ndufa6/Lhpp/Ndufa13/Cox8a/Atp5mf/LOC100363268/COX2/Ndufa1/Ndufa11/Cox7b/Uqcr10/	12
Huntington disease	15/296	202/8567	0.004	0.106	Sod1/Cox4i2/Ndufa6/Hap1/Ap2s1/Ndufa13/Cox8a/LOC100363268/COX2/Ndufa1/Bdnf/Ndufa11/Gpx1/Cox7b/Uqcr10/	15
Platelet activation	11/296	129/8567	0.005	0.112	Prkg2/Pik3r6/Fgb/Nos3/NEWGENE_621351/Adcy4/Rasgrp2/Fga/Fgg/Adcy9/LOC100361457/	11
Cardiac muscle contraction	8/296	81/8567	0.007	0.139	Cacng4/Cox4i2/Atp1b2/Cox8a/COX2/Tnnt2/Cox7b/Uqcr10/	8
Apelin signaling pathway	11/296	141/8567	0.010	0.183	Gnb2/Apln/Pik3r6/Jag1/Aplnr/Nos3/Plat/Adcy4/Ccnd1/Adcy9/Nos2/	11
Gap junction	8/296	88/8567	0.011	0.193	Prkg2/Tubb3/Adcy4/Tuba1c/Tubb4a/Tuba8/Adcy9/Tuba1b/	8
Thyroid hormone synthesis	7/296	72/8567	0.012	0.197	Atp1b2/Ttr/Duox2/Adcy4/Pax8/Gpx1/Adcy9/	7
Tight junction	12/296	170/8567	0.015	0.229	Cldn15/LOC103694903/Rab13/Myh14/Ccnd1/Tuba1c/Tiam1/Cldn5/Cttn/Tuba8/LOC100361457/Tuba1b/	12
Parkinson disease	11/296	152/8567	0.016	0.236	Cox4i2/Ndufa6/Slc6a3/Ndufa13/Cox8a/LOC100363268/COX2/Ndufa1/Ndufa11/Cox7b/Uqcr10/	11
Phagosome	13/296	198/8567	0.020	0.272	Sec61g/Sec61b/Cyba/Tubb3/Tuba1c/RT1-A2/C3/Tubb4a/Tuba8/RT1-CE10/LOC108348139/LOC100361457/Tuba1b/	13
Non-alcoholic fatty liver disease (NAFLD)	11/296	159/8567	0.022	0.286	Mlxip/Cox4i2/Ndufa6/Ndufa13/Cox8a/LOC100363268/COX2/Ndufa1/Ndufa11/Cox7b/Uqcr10/	11

of unsaturated fatty acids (**Supplementary Figure 1**). Thus, a maternal HF diet/obesity for dams can remodel the placenta and shape the gut microbiome to lipid dysmetabolism.

Fetal liver was selected for further study because the placnetal transcriptoms of the dams under the HF diet/obesity exhibited the NAFLD pathway. The fetal liver exhibited changes in the fatty liver and an increase in oxidative stress. In humans, the LPL gene expression and LPL activity of liver were higher in obese patients than in controls (44). Increased LPL activity could enhance the ability of hepatocytes to capture circulating triglycerides, leading to steatosis typically being observed in these patients. In mammals, glucose is converted into citrate in the mitochondria. Citrate is transported into the cytosol and cleaved into acetyl-CoA and oxaloacetate by ATP citrate lyase. ACC then carboxylates acetyl-CoA into malonyl-CoA. The FAS agglomerates malonyl-CoA and acetyl-CoA into a long-chain

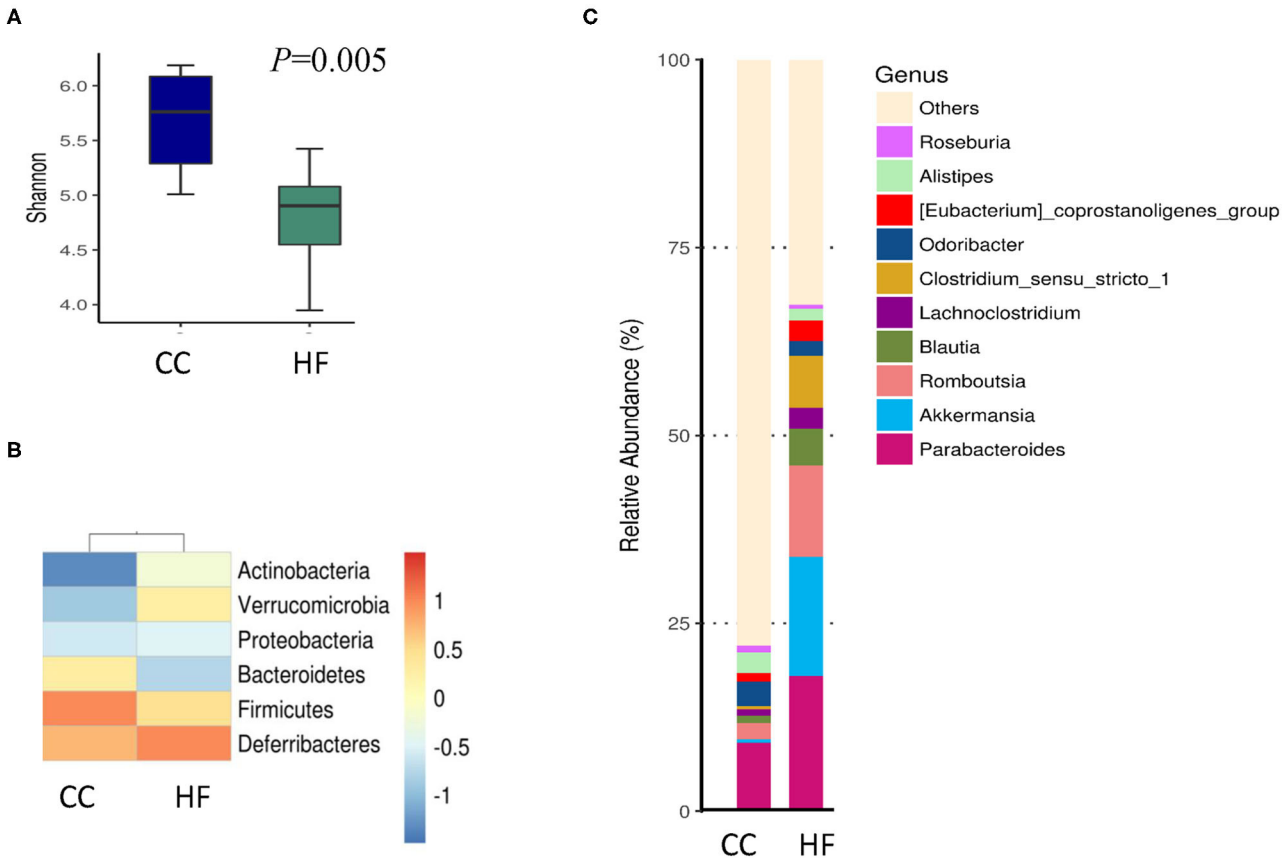

FIGURE 6 | Effect of high-fat diet on composition of gut microbiota in dams. **(A)** Lower alpha diversity in high-fat diet (HF) rats as compared with control diet (CC) rats. *$P < 0.05$. **(B)** Phylum and **(C)** genus classification of gut microbiota from dams on CC or HF. Column plot indicating genus class with a coverage of >95% ($n = 6$ for each group). The most abundant spectra are listed.

fatty acid (45). In our study, we observed a higher gene expression of LPL and ACC isoform 1 in fetal liver associated with maternal HF diet/obesity. Because disrupted hepatic metabolism and steatosis occur before any differences in body weight or body composition are observed (46), the dysregulation of hepatic lipid metabolism may be responsible for the programming of subsequent metabolic diseases in offspring with maternal HF diet/obesity.

Increased 8-OHdG levels in the placenta and fetal liver under a maternal HF diet suggest increased oxidative stress in both organs (47). Increased placental oxidative stress in maternal obesity is considered to lead to poor neonatal outcomes (48). The upregulation of nicotinamide adenine dinucleotide phosphate oxidase 2, a major source of reactive oxygen species, was suggested to be responsible for oxidative stress (49). Furthermore, oxidative damage marker significantly increased in the offspring liver with maternal HF diet/obesity. Reduced levels of glutathione peroxidase-1, the enzyme involved in antioxidation, was suggested to cause fatty liver in the offspring of mothers with obesity (46).

Among SCFAs, acetate, propionate, and butyrate are the most abundant (≥95%), with an approximate molar ratio of 3:1:1 (18, 50). Most SCFAs are produced through gut microbial anaerobic fermentation, and only a small portion is absorbed directly from food. Thus, SCFAs are produced depending on diet, microbiome constitution, and residence time in the intestinal tract (7, 8). Accumulating evidence indicates that SCFAs are key to the maintenance of health and crucial in disease development, including with regard to intestinal integrity, inflammation presentation, immune modulation, and metabolic homeostasis (18). Regarding the FFAR, both GPR41 and GPR43 are closely related to metabolic processes and have become potential targets for the treatment of type 2 diabetes, cardiovascular disease, and metabolic syndrome (19). GPR43 can stimulate insulin secretion and inhibit the apoptosis of islets cells (51). Moreover, GPR43 modulates the peptide YY–glucagon-like peptide-1 pathway for the intestine to achieve metabolic homeostasis (52). Increased expression levels of GPR41 and GPR43 in fetal membranes and placenta were noted after labor onset. Through GPR43, propionate can reduce the LPS-induced neutrophil chemotaxis and IL-8 secretion of amnion explants (53). In our study, we found that both serum propionate level and placental GPR43 expression decreased in the HF group. Maternal obesity or HF diet is usually associated

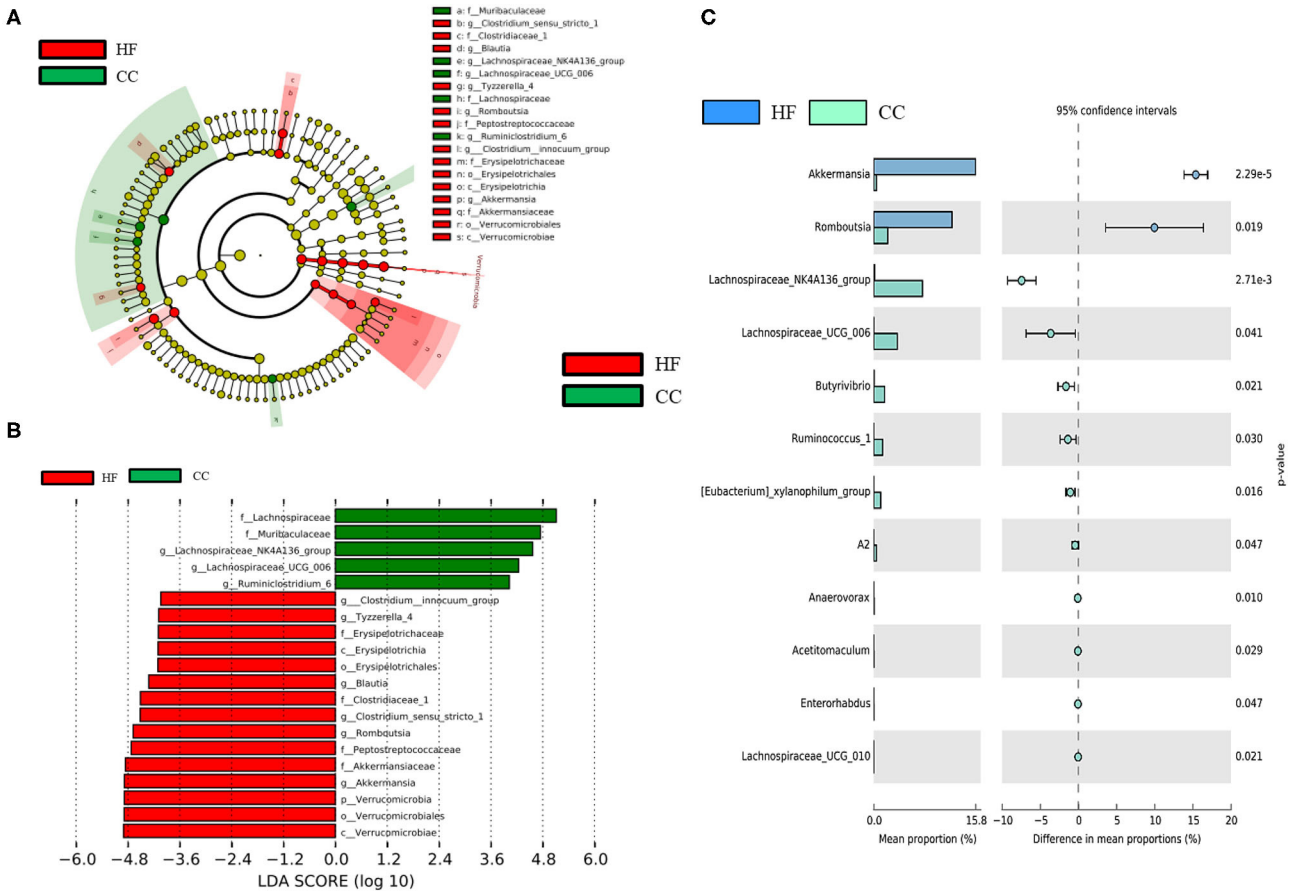

FIGURE 7 | LEfSe analysis identified crucial bacteria associated with exposure to a high-fat diet (HF) in rats. **(A)** Cladogram representing taxa at all phylogenetic levels indicated predominant bacteria associated with a control diet (CC) and HF diet. For the cladogram section, the circle from the inside to the outside indicates the classification stratum from the gate to the genus. Each small circle indicates a classification at that stratum, and the diameter of the circle represents its relative abundance. The colors indicate significant differences in the biomarkers and grouping between the two groups. **(B)** For the LDA distribution histogram, taxa that reached a linear discriminant analysis score ($\log_{10}$) of >2.0 were highlighted and labeled. **(C)** At the genus level, a significant difference was observed between the CC and HF groups. ($n = 6$ for each group).

with placental inflammation, which presents as an increase in proinflammatory cytokine abundance and macrophage accumulation (54, 55). Thus, the change in the propionate–GPR43 axis could be a factor that heightens inflammation associated with an HF diet.

Another study reported that pregnant C57BL/6J dams have a higher proportion of Clostridium and Akkermansia but a lower proportion of Lachnospira and Ruminococcus genera when an HF diet is adopted (13). Our results partly agree with this finding. Furthermore, an abundance of Verrucomicrobia (from phylum to order) was noted in our study. Although a lower proportion of Lachnospira and Ruminococcus genera was suggested to cause hypo-butyrate in dams owing to their butyrate-producing ability (13), this was not the case in our study. Our HF diet/obesity dams exhibited lower plasma propionate levels but similar butyrate levels compared with the control dams. Owing to complex interactions among different genera, the production of SCFAs may not be completely explained by the relative abundance of a few genera alone.

On the basis of the tail-cuff measurement, the HF diet administered before mating and during pregnancy resulted in higher systolic BP than did the chaw diet. Our results are consistent with those of several reports that indicating that an HF diet during pregnancy causes higher BP in animals on the basis of tail-cuff measurements (56–58). By contrast, one study reported no difference in mean artery pressure, measured using a carotid catheter, between rats receiving an HF diet and those receiveing a control diet during pregnancy (59). This inconsistency in the results was partially explained by the difference in measurement methods.

One limitation of this study was maternal obesity being induced by an HF diet. Whether the gut microbiomes were similar to those in other obesity models, such as high-fructose or high-glucose models, was unknown. One study examined the changes in the gut microbiome of rats with obseity induced by an HF + high-fructose (HFF) diet and an HF + high-sucrose (HFS) diet. At the species level, a significant increase in Limosilactobacillus reuteri and Bacteroides fragilis in the

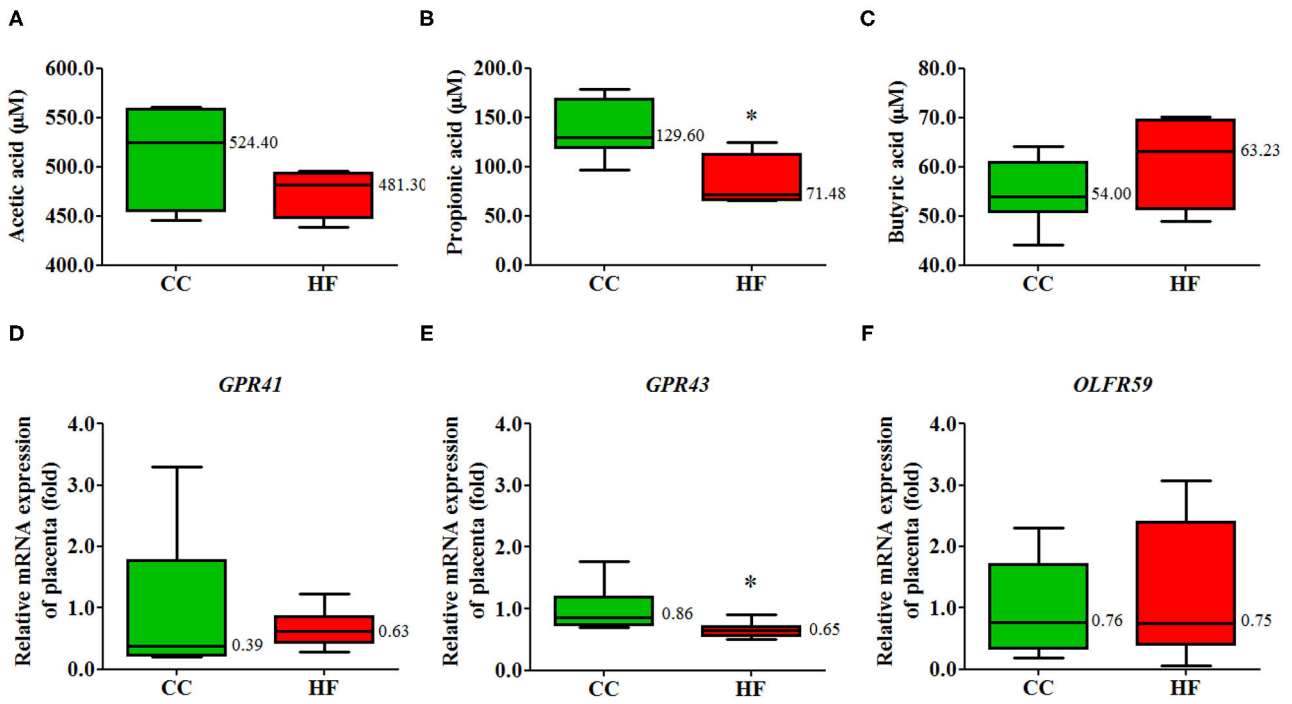

FIGURE 8 | Plasma short-chain fatty acid (SCFA) levels and gene expression of corresponding receptors in placenta tissue. Plasma **(A)** acetic acid, **(B)** propionic acid, and **(C)** butyric acid levels of the rats with high-fat (HF) diet or control (CC) diet determined using gas chromatography. The mRNA expression of **(D)** *GPR41*, **(E)** *GPR43*, **(F)** *OLFR59* in placental tissue. The housekeeping gene was 18S ribosomal RNA, and mRNA expression is presented in terms of relative fold ($n = 6$ for each group). *$P < 0.05$. The median was shown. GPR, G-protein-coupled receptor; OLFR59, olfactory receptor 59.

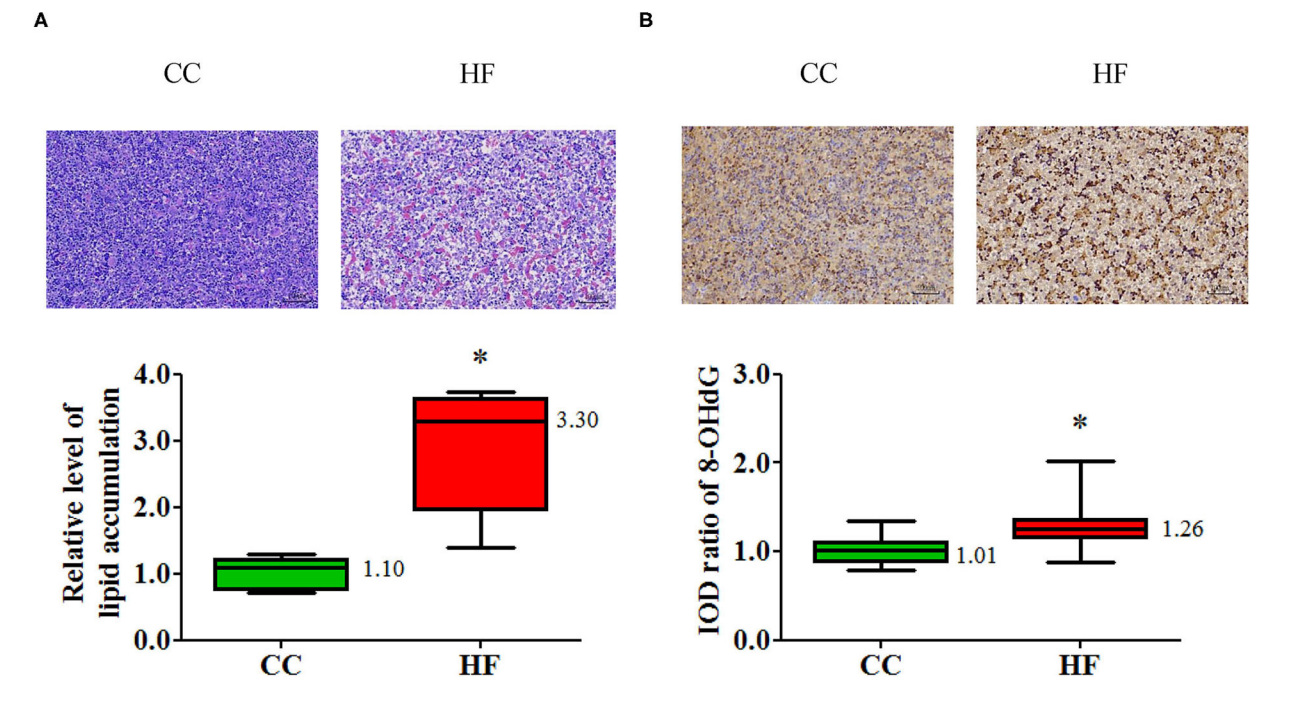

FIGURE 9 | Histological changes in fetal liver associated with a maternal high-fat (HF) diet. **(A)** Degree of fetal hepatic steatosis increased with a maternal HF diet and manifested as increased vacuolation in H&E staining. **(B)** Fetal liver oxidative stress was higher with a maternal HF diet compared with a maternal control diet (CC). Oxidative stress was determined based on 8-hydroxy-2-deoxyguanosine (8-OHdG). *$P < 0.05$. ($n = 6$ for each group) The median was shown.

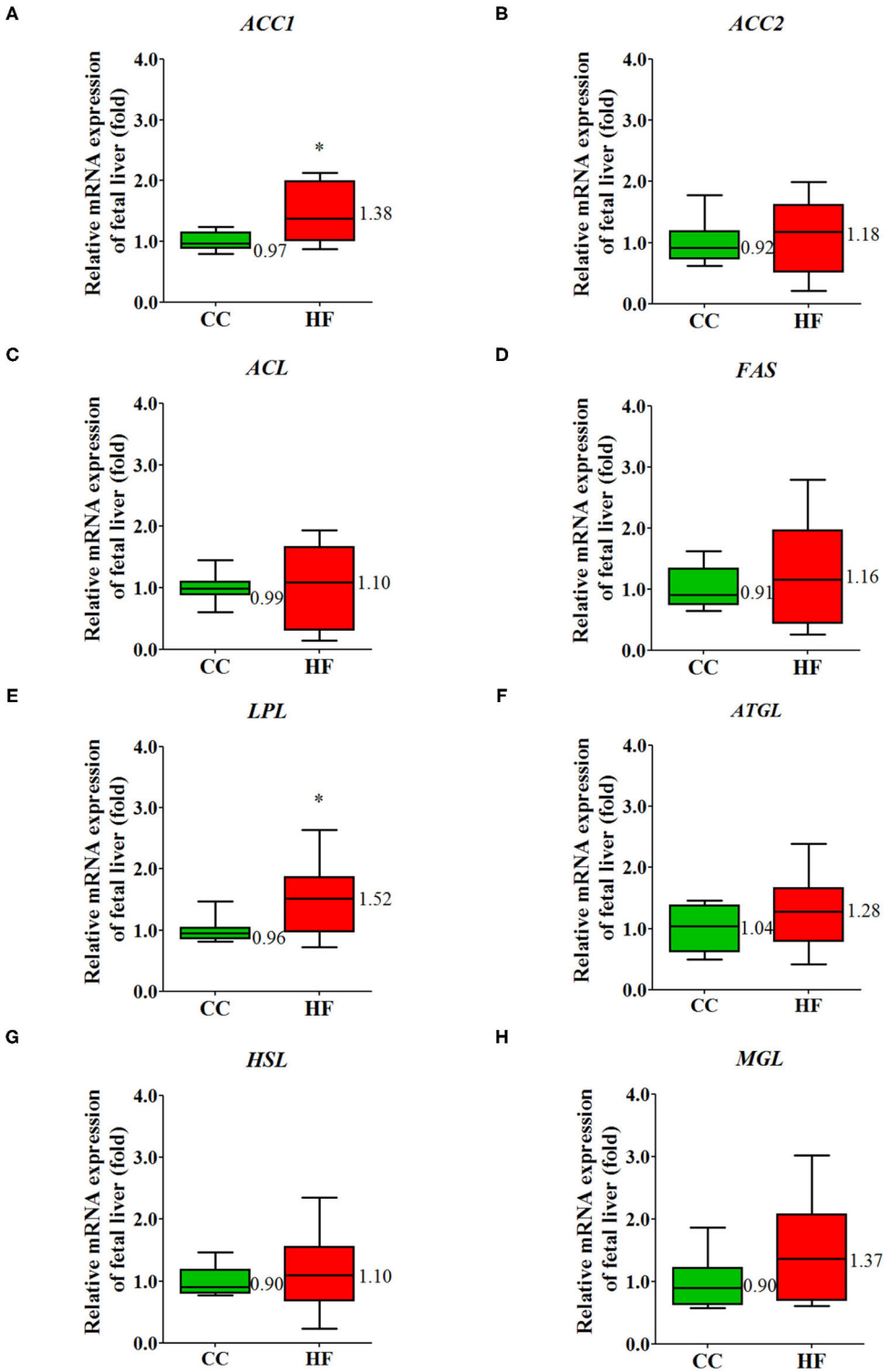

FIGURE 10 | Maternal high-fat diet disrupts the key enzymes in the lipid metabolism of fetal liver. The mRNA expression of target gene. **(A)** *ACC1*, **(B)** *ACC2*, **(C)** *ACL*, **(D)** *FAS*, **(E)** *LPL*, **(F)** *ATGL*, **(G)** *HSL*, **(H)** *MGL*. The housekeeping gene was GAPDH, and mRNA expression is presented in terms of relative fold. *$P < 0.05$ ($n = 8$ for each group). The median was shown. ACC1, acetyl-CoA carboxylase 1; ACC2, acetyl-CoA carboxylases 2; ACL, ATP-citrate synthase; FAS, fatty acid synthase; LPL, lipoprotein lipase; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase.

HFF group and an increase in Brachycybe producta in the HFS group were observed (60). Thus, other obesity models may have different gut mocribiome profiles.

CONCLUSIONS

These findings jointly suggest that maternal HF diet or obesity shapes the composition of the maternal gut microbiota and remodels placenta, resulting in placental oxidative stress increase and lipometabolism disruption in fetal liver, which may ultimately contribute to the programming of offspring obesity.

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: NCBI SRA; PRJNA746978.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal, the Institutional Animal Care, and Use Committee of Chang Gung Memorial Hospital (Approval Number: 2019053001).

AUTHOR CONTRIBUTIONS

Y-WW, H-RY, J-MS, M-MT, Y-LT, and L-TH contributed to design the work. H-RY, C-CC, I-CL, and Y-JL contributed to data acquisition. H-RY, C-CT, Y-JL, K-AC, and L-TH performed data analysis and interpretation. Y-WW, H-RY, JMS, M-MT, Y-JL, and C-CT drafted the manuscript. Y-WW, H-RY, Y-JL, C-CT, K-AC, and L-TH finalized the article. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.736944/full#supplementary-material

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frontiers in Nutrition

ORIGINAL RESEARCH
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Lactational High Fat Diet in Mice Causes Insulin Resistance and NAFLD in Male Offspring Which Is Partially Rescued by Maternal Metformin Treatment

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Maternal metabolic disease and diet during pregnancy and lactation have important implications for the programming of offspring metabolic disease. In addition, high-fat diets during pregnancy and lactation can predispose the offspring to non-alcoholic fatty liver disease (NAFLD), a rising health threat in the U.S. We developed a model of maternal high-fat feeding exclusively during the lactation period. We previously showed that offspring from dams, given lactational high-fat diet (HFD), are predisposed to obesity, glucose intolerance, and inflammation. In separate experiments, we also showed that lactational metformin treatment can decrease offspring metabolic risk. The purpose of these studies was to understand the programming implications of lactational HFD on offspring metabolic liver disease risk. Dams were fed a 60% lard-based HFD from the day of delivery through the 21-day lactation period. A subset of dams was also given metformin as a co-treatment. Starting at weaning, the offspring were fed normal fat diet until 3 months of age; at which point, a subset was challenged with an additional HFD stressor. Lactational HFD led male offspring to develop hepatic insulin resistance. The post-weaning HFD challenge led male offspring to progress to NAFLD with more severe outcomes in the lactational HFD-challenged offspring. Co-administration of metformin to lactating dams on HFD partially rescued the offspring liver metabolic defects in males. Lactational HFD or post-weaning HFD had no impact on female offspring who maintained a normal insulin sensitivity and liver phenotype. These findings indicate that HFD, during the lactation period, programs the adult offspring to NAFLD risk in a sexually dimorphic manner. In addition, early life intervention with metformin via maternal exposure may prevent some of the liver programming caused by maternal HFD.

Keywords: lactation, dietary fat, developmental programming, NAFLD, metformin

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INTRODUCTION

The field of developmental programming has provided irrefutable evidence that maternal exposures shape the metabolic health of the offspring for a lifetime (1, 2). Exposures during critical windows of development can permanently alter maturing offspring tissues, leading to increased susceptibility to metabolic decompensation. The lactation period is one critical susceptibility window; during which, alterations in milk composition can permanently change ongoing organ development and maturation, thus altering offspring organ function (3). Both maternal diet and maternal metabolic health during the lactation period play a role in transmitting this risk of metabolic syndrome to offspring (4, 5).

Previous studies have used cross fostering techniques and maternal diet isolated to the lactation period to show that, even in the absence of maternal obesity, diet composition has an ability to shape offspring metabolic outcomes (4, 6, 7). We and others have shown that feeding mouse dams a 60% high-fat diet (HFD) exclusively during the lactation period predisposes their offspring to obesity, insulin resistance, and glucose intolerance (4, 8). This programming is sexually dimorphic in rodents with males experiencing increased metabolic consequences and female offspring being relatively spared.

Non-alcoholic fatty liver disease marked by hepatic steatosis may progress from simple steatosis to non-alcoholic steatohepatitis (NASH), which is expected to become the leading cause of liver transplantation in the future (9). Dietary exposures play a role in the pathogenesis and progression of this liver disease. Overnutrition promotes obesity and insulin resistance, stimulating the release of fatty acids from adipose tissue into the blood and, eventually, the liver (10). In the process of *de novo* lipogenesis, carbohydrates are converted to free fatty acids in the liver by enzymes, such as SREBP1c, Acetyl CoA Carboxylase (ACC), and Fatty Acid Synthase (FAS). Overconsumption of carbohydrates and fat may lead to the accumulation of free fatty acids in the liver, and excess free fatty acids generate lipotoxic products, followed by a vicious cycle of hepatocellular injury, inflammation, and repair that can lead to fibrosis and hepatocellular carcinoma (10). With the rising prevalence of non-alcoholic fatty liver disease (NAFLD) and NASH (11), it is important to understand how life stages such as the lactation period can both contribute to the risk of lifetime metabolic liver disease and be targeted for intervention.

Lactational programming studies show that the neonatal liver is vulnerable to stressors. In response to a variety of stressors, offspring livers can be compromised. High carbohydrate diets fed to pups by gastrostomy, for example, led to increased adult liver fat (12). Lactational HFD also led to increased liver triglycerides and steatosis in several models (13–15). Small litters used to model early post-natal overnutrition show increased liver oxidative stress, with microsteatosis followed by insulin resistance (16, 17). The relationships between liver dysfunction and offspring insulin resistance in these models have not been evaluated in detail.

The objective of the current study is to dissect out the contributors to the insulin-resistant phenotype we detected in male offspring in previous studies. We used hyperinsulinemic, euglycemic clamps to understand the different tissue contributions to the whole-body phenotype. We also designed an intervention study to understand if the use of metformin during lactation, while the dam is consuming an HFD, could rescue the offspring metabolic programming. Metformin is the most commonly used oral anti-diabetes medication and can lower circulating insulin levels (18). It has been studied during the critical windows of pregnancy and lactation with some evidence for improved offspring health, following this exposure, although the results vary, depending on maternal health and dosing strategy (19–23). The lactation period is an underutilized window for intervention, and pharmacologic interventions may be more readily adopted by mothers than making a change in dietary fat consumption.

MATERIALS AND METHODS

Animals

Mice were housed in ventilated cages in a facility with a 12-h light/dark cycle and provided with *ad libitum* access to food and water. All animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committee. Two-month-old virgin C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and allowed to acclimate for 2 weeks prior to breeding. Upon parturition, a subset of dams was switched onto a 60% HFD (D12492 Research Diets New Brunswick, NJ, USA); remaining dams were maintained on 5001 (Normal Diet/ND) (13.5% kCal from fat, LabDiet, St. Louis, MO, USA) for the duration of the lactation period. For metformin rescue experiments, dams receiving HFD after parturition were also given sterile water bottles containing 3 mg/ml metformin-HCl (Spectrum Chemical, New Brunswick, NJ, USA). Bottles were changed weekly up to post-natal day 21. On post-natal day 21, offspring were weaned onto normal diet (ND). At 3 months of age, half of the offspring from the Ctrl or HFD dams were placed onto HFD for an additional 3 months. Experimental offspring generated fell into five groups: maternal normal diet (Ctrl PN), maternal normal diet with adult high-fat diet rechallenge (Ctrl PN + HFD), maternal high-fat diet (HFD PN), maternal high-fat diet with adult high-fat diet rechallenge (HFD PN + HFD), and maternal high-fat diet plus metformin (HFD + Met PN). Only offspring from litters with five to nine pups were used for the experimental groups, as this represents an average litter size in our colony. All experiments on adult offspring, with the exception of the hyperinsulinemic, euglycemic clamp, were performed on multiple, independent cohorts to demonstrate reproducibility. In one cohort using the same groups, non-fasted offspring were euthanized via CO_2 inhalation at post-natal day 16 for blood and tissue collection. All other offspring were euthanized via CO_2 inhalation at 6 months of age, and liver samples were either fixed in 4% formaldehyde or snap frozen and stored at −80°C for downstream analysis.

Circulating Insulin Levels

At 2 months of age, offspring were fasted for 6 h, and blood was collected by tail snip and centrifuged to separate the serum.

Blood from non-fasted, 16-day-old offspring was collected by cardiac puncture and centrifuged to separate the serum. Serum samples were analyzed for insulin concentration using a Mouse Ultrasensitive Insulin ELISA (ALPCO, Salem, NH, USA). The sensitivity for this test is 0.115–6.9 ng/ml with a reported coefficient of variation of <9.3%.

Insulin Tolerance Test

Mice were subjected to insulin tolerance tests at 5 months of age (Ctrl PN $n = 6$, HFD PN $n = 15$, HFD + Met PN $n = 9$). Mice were fasted in the morning for 6 h prior to testing. Each mouse was administered 0.75 units/kg of insulin lispro via intraperitoneal injection. Blood glucose was measured after fasting, prior to injection, and at 15, 30, 60, and 90 min after injection using a Bayer Contour glucometer (Bayer AG, Leverkusen, Germany). The area under the curve was calculated for each animal as the sum of glucose values during the experiment. To test initial responsiveness to insulin administration, we calculated individual rates of fall by limiting the data to the first 45 min of the experiment, and then regressing log glucose by time for each animal. This resulted in an individual rate and intercept for each animal. Slopes were then calculated by multiplying the exponentiated intercept by the rate, generating rate estimates in mg/dl.

Hyperinsulinemic-Euglycemic Clamp

The hyperinsulinemic-euglycemic clamp (HEC) study was carried out by the University of Michigan Animal Phenotyping Core. Male Ctrl PN ($n = 9$), HFD PN ($n = 12$), and HFD + Met PN ($n = 11$) offspring aged 3 months exposed to either HFD or ND during the lactation period underwent surgical implantation of indwelling catheters in the right carotid artery and right jugular vein. The catheters were subcutaneously tunneled and exteriorized at the back of the neck. After recovering from implantation surgery (5 days), conscious and unrestrained animals underwent a 5-h fast before initiation of the clamp study. A priming dose of [3-H^3] glucose (1 μCi) was administered beginning at 90 min before insulin administration ($t = -90$ min), followed by infusion of .05 μCi per minute for 90 min. The insulin clamp began at $t = 0$ with a prime-continuous infusion (16 mU/kg bolus, followed by 4.0 mU/kg/min) of human insulin (Novo Nordisk). The infusion of [3-H^3] glucose was increased to 0.10 μCi/min for the rest of the experiment. Blood samples were collected via the carotid artery at $t = -10$ and 120 (for basal and final insulin) as well as 80, 85, 90, 100, 110, and 120 min for glucose specific activity.

Continuous glucose infusion (50% glucose in phosphate-buffered saline) began at time = 0 min and continued throughout the 120-min procedure. Blood glucose was monitored with a glucometer (Accu-check, Roche, Basel, Switzerland) every 10 min, and adjustments were made to the glucose infusion rate for each animal to remain euglycemic (120–130 mg/dl). To determine tissue-specific uptake of glucose, a bolus injection of [1-14C]-2 deoxyglucose (10 μCi) was administered at $t = 78$ min. At the end of the clamp ($t = 120$), animals were anesthetized with an intravenous infusion of sodium pentobarbital. The liver, heart, gastrocnemius, brown adipose depot, inguinal white adipose depot (subcutaneous), and epididymal adipose depot (visceral) were collected, weighed, and snap-frozen in liquid nitrogen and kept at −80°C for later use. [14C]2-Deoxyglucose-6-phosphate was then determined from tissues using a liquid scintillation counter. Hepatic tissue samples collected during the HEC were used for determination of *de novo* lipogenesis (DNL) by calculating disintegrations per minute per mg of lipid extracted from the liver tissue. Serum non-esterified fatty acid (NEFA) levels were determined from samples collected before and during the clamp study using the NEFA-HR (2) kit (Catalog No. 276-76491; Wako Diagnostics) in accordance with manufacturer's guidelines. Percent suppression of NEFA and EGP was calculated by subtracting the value for either measure during the clamped portion of the experiment from the basal value, and then dividing by the basal value and multiplying by 100.

Picro-Sirius Red Staining

Picro-sirius red staining (also called Sirius red staining) highlights fibrosis by staining collagen (Collagen I and III fibers) fibers in formaldehyde-fixed, paraffin-embedded liver tissue sections (24). Sirius red-stained collagen appears red by light microscopy. Tissue sections were deparaffinized and rehydrated followed by staining in Picro-sirius Red solution for 1 h. Slides were dehydrated in three changes of 100% ethanol, followed by xylene and mounted with Permount medium. The images were taken on an Olympus microscope (Olympus Corporation of the Americas, Center Valley, PA, USA) and analyzed using freely available Image J software. Five sections were examined for each animal.

Hematoxylin and Eosin Staining

Formaldehyde-fixed, paraffin sections of liver were cut at 5 μm. All tissues were stained as described previously (20).

Immunofluorescent Staining

Formaldehyde-fixed, paraffin embedded liver samples were sectioned at 6 μm. After deparaffinization and antigen retrieval, the slides were blocked and permeabilized with 5% BSA PBS with 0.1% Triton × 100 for 20 min at room temperature. The slides were incubated with a primary antibody at 4°C overnight. Primary antibodies used were polyclonal anti-caveolin (Cell Signaling Technology Cat # 3238 Danvers, MA, USA) and monoclonal anti-Mac2 (Galectin 3, eBioscience M3/38) (Thermo Fisher Scientific, San Diego, CA, USA). This was followed by a secondary antibody, fluorescein isothiocyanate anti-rabbit (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Slides were mounted with a Vectashield mounting medium containing DAPI mounting media. The sections were imaged on an Olympus microscope.

Hepatic Triglyceride Quantification

The total triglycerides in liver were analyzed by Thermo Scientific™ Triglycerides Reagent (TR22421), following the manufacturer protocol. The triglycerides in liver were extracted using the previously described protocol (25). The triglyceride

results were normalized to the mass of the liver tissue initially used for assay, as previously reported (26).

Microarray Methods

For RNA microarray studies, an Affymetrix Plus WT GeneAtlas2.1 ST array platform was used. Biotinylated cDNA was prepared according to the Affymetrix Plus WT kit protocol (GeneChip® WT Plus Reagent Kit Manual P/N 703174 Rev. 2) from 400 ng total RNA. Following the labeling procedure, 2.76 ug of cDNA was hybridized at 48°C on Mouse Gene 2.1 ST and was washed and stained using the Affymetrix Gene Atlas system (software version 2.0.0.460). Peg Arrays were scanned using the Affymetrix Gene Atlas system (software version 2.0.0.460). RMA was used to fit log2 expression values to the data using the oligobioconductor package in R version 3.4.3. For gene set enrichment analysis, transcript-level data were reduced to gene-level data via tximeta (27) and txiimport (28) prior to analysis by DESeq2 (29) v1.28.1. For gene set enrichment analyses, we used ClusterProfiler v3.16 after conversion of mouse genes to human genes and ranking by fold change (30, 31). Data are available from GEO at accession No. GSE182071.

RNA Extraction and qRT-PCR

Ribonucleic acid was extracted from frozen liver samples using an RNeasy Mini Kit (QIAGEN, Gaithersburg, MD, USA) according to the manufacturer's instructions. cDNA was generated using a high-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA), followed by analysis using real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA). The gene *Gapdh* was used as an internal control. A list of primer sequences can be seen in **Table 1**.

Western Blotting

Liver tissue collected at the end of the hyperinsulinemic-euglycemic clamp was snap frozen in liquid nitrogen and stored at −80°C. Liver tissue was cut on dry ice and homogenized in a RIPA buffer. Samples were run on SDS-PAGE gel and transferred overnight onto nitrocellulose membranes. Total protein was assessed via REVERT stain (Li-COR Biosciences, cat # 926-11011) and was used to normalize target signals [AKT and phosphor-AKT(ser473)]. Nitrocellulose membranes were then blocked in 2% Bovine Serum Albumin for 1 h before being incubated with a primary antibody. Primary antibodies used were P-AKT: Cell Signaling Technology, Catalog # 4060S (Danvers, MA, USA) (rabbit) and AKT (pan): Cell Signaling Technology, Catalog # 2920S (mouse) (Danvers, MA, USA). Blot was then washed in TBST before incubation with a secondary antibody. Blots were imaged using Li-COR scanner and quantified using Image Studio. All protein targets were normalized to total protein.

Statistical Analysis

Insulin Tolerance Test

Statistical analyses for insulin tolerance testing were completed using mixed linear effects models, with fixed effects of time and experimental treatment, and random effects of individual mouse ID and maternal mouse ID. Further analysis on the initial rate of drop in response to insulin administration was conducted by limiting the dataset to observations before 20 min. Then, linear models were constructed with effect of the group and of time and the interaction thereof. The coefficient of the interaction estimate was then evaluated. Models with $p < 0.05$ were considered to be statistically significant.

Hyperinsulinemic Euglycemic Clamp

The assumption of euglycemia was assessed via mixed linear modeling and was found to be similar between all control and HFD groups. Hyperinsulinemia assumption was tested by two-way ANOVA. Analysis of glucose infusion rate (GIR) was conducted by use of mixed linear effects modeling, with fixed effects of time and experimental group and random effect of individual animal IDs. The area under the curve was determined as the sum of GIR values for the duration of the experiment and was assessed via pairwise testing. Values were assessed for normality using Shapiro–Wilk tests and equivalence of variance using Levene's test. When values failed tests of normality, non-parametric tests were used instead of Student's *T*-test.

Other Results

Data are shown as mean ± standard error of the mean. Data were checked for normality and equal variance using the Shapiro–Wilk and Brown–Forsythe tests. Two-tailed *T*-tests, Welch's Student's *T*-test or Mann–Whitney *U*-tests were used as appropriate to compare results from two groups. In the metformin experiments where three groups were analyzed, one-way ANOVA was performed to analyze for differences between treatment groups, followed by pairwise *T*-tests. Significance was determined as $p < 0.05$. Statistical analyses were performed using GraphPad Prism 9.0 software.

RESULTS

An Offspring Metabolic Profile After Lactational Exposure to HFD

The experimental groups timeline are shown in **Figures 1A,B**. We first examined offspring insulin sensitivity during and after the lactational HFD exposure. The groups we examined were offspring of dams-fed ND during lactation (Ctrl PN) and offspring of dams-fed HFD during lactation (HFD PN). Male and female data from birth to weaning were combined, as we do not observe sex differences until mice reach adulthood. As we and others have previously described, HFD PN offspring have an elevated circulating insulin level at post-natal day 16 (P16) (**Figure 2B**) and heavier weights during the lactation period on days 7, 14, and 21 ($p < 0.0001$ at each timepoint) (**Figure 6A**) (8, 32). Despite this, we did not detect a change in the offspring liver weights during the lactation period (**Figure 2A**). There was also no difference in circulating triglyceride levels between Ctrl PN and HFD PN pups at P16 (data not shown). Fasting insulin levels at 2 months were unchanged in both male and female HFD PN offspring fed ND (**Figures 2C,D**). Insulin tolerance testing was conducted in Ctrl PN and HFD PN offspring at 5 months of age (**Figures 2E,F**). Male HFD PN offspring had similar fasting blood glucose levels compared to control males

TABLE 1 | Sequences for qPCR primers.

Gene	Forward 5′-3′	Reverse 5′-3′	NCMI ID
Angptl4	GGA CCT TAA CTG TGC CAA GA	CGT GGG ATA GAG TGG AAG TAT TG	
Cyp4a14	TCT CAT CTT TCT GCC CTC ATT TC	CAG TGG CTG GTC AGA GTT AAA G	
Cyp7a1	CAC CTT GAG GAT GGT TCC TAT AA	TCA AAG GGT CTG GGT AGA TTT C	
Fabp4	GTG AAG AGC ATC ATA ACC CTA GAT	CAC GCC TTT CAT AAC ACA TTC C	
Fbp1	TTC ACC GCA CTC TGG TAT ATG	CGG CCT TCT CCA TGA CAT AA	NM_09395.3
Foxo1	CGT GCC CTA CTT CAA GGA TAA G	GCA CTC GAA TAA ACT TGC TGT G	
G6pc	CAA CAG CTC CGT GCC TAT AA	TAG CAA GAG TAG AAG TGA CCA TAA C	NM_008061.4
Gapdh	AAC AGC AAC TCC CAC TCT TC	CCT GTT GCT GTA GCC GTA TT	GU214026.1
Pck1	GGC ACC TCA GTG AAG ACA AA	CGA TGA CTT CCC AGT AAA CA	NM_011044.3
Plin2	GAA GGA TGT GGT GAC GAC TAC	TCA CTG CTC CTT TGG TCT TAT C	
Ppara	CGG TGT GTA TGA AGC CAT CT	TAA GGA ACT CGC GTG TGA TAA A	
Zbtb16	CGT CTG TGG ATC TGA ACT GTA TC	AGG AAG GAA GGA AGG AAG GA	

NCMI IDs are listed when available.

($p = 0.6$, **Figure 2H**) but had a 16% greater area under the curve after insulin administration, suggesting some level of insulin resistance in this group ($p = 0.003$, **Figure 2G**). However, mixed linear modeling failed to reach statistical significance ($p_{\text{diet}} = 0.075$). There was no difference between groups in the rate of fall in blood glucose in the first 15 min of the ITT (**Figure 2I**). Female offspring had no difference in insulin response between groups (**Figure 2F**).

Hyperinsulinemic Euglycemic Clamp

To further assess the differences in glucose homeostasis identified by ITT in male offspring, a hyperinsulinemic euglycemic clamp was conducted at 3 months of age in one cohort of male offspring. The glucose infusion rate (**Figure 3A**), representing whole body insulin sensitivity, was similar between Ctrl PN and HFD PN males, consistent with modest insulin resistance. The glucose infusion rate area under the curve (**Figure 3B**) did not differ between the Ctrl PN and the HFD PN group ($p = 0.5$), nor did insulin concentration differ between groups at basal ($p = 0.6$) or clamped ($p = 0.7$) conditions (**Figure 3C**). Insulin turnover was similar between groups. HFD PN males had 37% higher glucose turnover at basal conditions ($p = 0.008$) and 30% higher when clamped ($p < 0.0001$; **Figure 3D**). Thus, there is greater glucose disposal in HFD PN males under both basal and insulinemic conditions. Endogenous glucose production (EGP) was 58% higher in HFD PN males at basal ($p = 0.008$) and 183% higher in clamped conditions ($p < 0.0001$; **Figure 3E**). Consistent with this, we found a 63% greater overall suppression of EGP achieved during the clamp in Ctrl PN males ($p < 0.0001$; **Figure 3F**). This suggests that HFD PN males developed insulin resistance of the liver, with impaired suppression of hepatic glucose production in response to insulin. We also found that *de novo* lipogenesis in response to insulin in the liver of HFD PN males was 22% lower than Ctrl PN males without reaching statistical significance ($p = 0.09$, **Figure 3G**), possibly indicating mild hepatic insulin resistance. Non-esterified fatty acids (NEFA) were 25% lower in basal conditions for HFD PN males ($p = 0.04$, **Figure 3H** basal conditions), indicating lower levels of lipolysis. The total suppression of NEFAs in the hyperinsulinemic state was 25% lower in HFD PN males; however, this did not reach statistical significance ($p = 0.08$, **Figure 3I**). It is plausible that these baseline differences in the amount of lipolysis in HFD PN males drive increased hepatic gluconeogenesis. These differences may also not be specific to the effects of insulin as NEFA levels were similar between Ctrl PN and HFD PN males under hyperinsulinemic conditions ($p = 0.99$, **Figure 3H** clamped conditions). We next examined liver-specific insulin signaling through western blotting in liver tissue collected immediately after the clamp by assessing AKT and phosphorylated (serine 473) AKT (**Figure 3J**) as a major readout. We found no reduction in insulin signaling in the livers of HFD males ($p = 0.4$), suggesting the insulin resistance apparent from the clamp study is not fully attributable to liver-specific AKT phosphorylation. We anticipated that the increased peripheral glucose disposal in HFD PN males (**Figure 3D**) would be evident in tissue-specific uptake of glucose. When individual tissues were analyzed, however, three of the five tissues showed significantly lower glucose uptake in HFD males, 25% lower in heart ($p = 0.003$), 29% lower in subcutaneous adipose ($p = 0.03$), and 37% lower in visceral adipose ($p = 0.02$, **Supplementary Figure 1**). This suggests that glucose turnover may have been elevated in a tissue that was not collected for analysis, potentially brain or liver tissues. Our interpretation is that this is unrelated to insulin signaling as the glucose turnover was elevated in both basal and hyperinsulinemic conditions. Taken together, we present evidence of some degree of insulin resistance at the liver, heart, and adipose tissue of male HFD PN offspring. However, the differences in baseline measures between Ctrl PN and HFD PN males may implicate a non-insulin dependent mechanism or tissue in the development of this phenotype.

Liver Pathology in Male High-Fat Diet Offspring

Due to the evidence of physiological liver insulin resistance on the hyperinsulinemic euglycemic clamp, we next focused on analysis of the liver morphology. We examined the livers in two

A

Group name	Maternal treatment in lactation	Offspring diet at weaning	Offspring diet at 12-24 weeks
Ctrl PN	Normal Diet	Normal Diet	Normal Diet
HFD PN	60% HFD	Normal Diet	Normal Diet
HFD+ Met PN	60% HFD + Metformin in drinking water 3mg/mL	Normal Diet	Normal Diet
Ctrl PN+ HFD	Normal Diet	Normal Diet	60% HFD (Re-challenge)
HFD PN+ HFD	60% HFD	Normal Diet	60% HFD (Re-challenge)

B

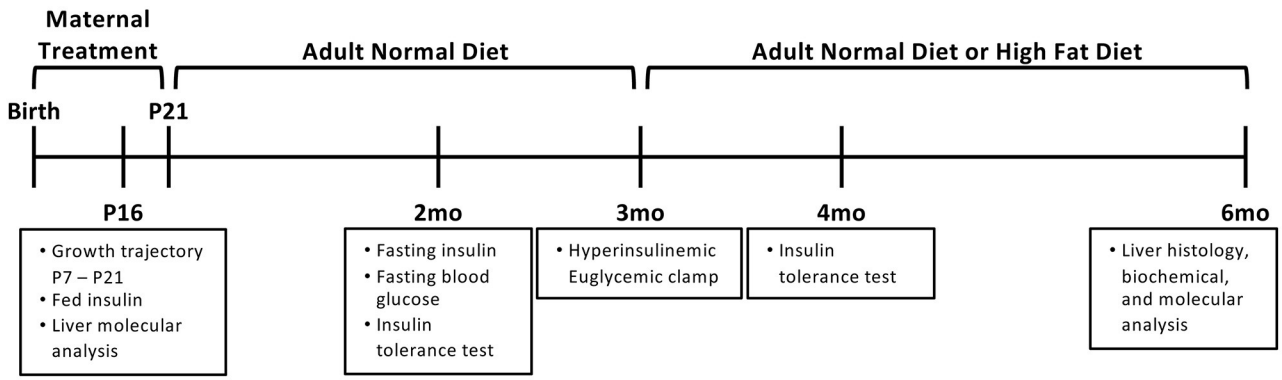

FIGURE 1 | Experimental schema for the experiments presented. **(A)** A scheme of diet conditions per group. **(B)** Experimental timeline. Ctrl, control; HFD, high-fat diet; PN, post-natal; Met, metformin.

separate experimental conditions. We first studied livers of male and female Ctrl PN and HFD PN offspring at 6 months of age that had been weaned onto and maintained on ND throughout life. In the other condition, we rechallenged the offspring in both Ctrl PN and HFD PN groups with a "second-hit" stressor of an HFD from 3 to 6 months of age. This "second-hit" is sometimes necessary to unmask programmed metabolic defects. There was no difference in whole liver mass in either male or female mice maintained on ND. After HFD rechallenge, we observed a significant increase in whole liver mass in male HFD PN offspring, which was not seen in female offspring (**Figures 4A,B**). We continued our liver morphology investigation in male mice, given the lack of differences in liver mass and insulin resistance in female mice reported here and previously (8). We found that male offspring from both Ctrl PN and HFD PN maintained on ND had no evidence of liver fat accumulation or fibrosis (**Figures 4C,D**). Pathologist examination of hematoxylin and eosin-stained liver specimens (**Supplementary Table 1**) showed no abnormalities both at post-natal day 16 and 6 months of age in the Ctrl PN and HFD PN groups. Male HFD PN + HFD offspring developed evidence of hepatic steatosis and fibrosis. Pathologist review of male Ctrl PN + HFD offspring showed that 4/7 had evidence of microvesicular steatosis with 1/7 also showing macrovesicular steatosis. HFD PN + HFD male offspring showed that 5/5 had evidence of both microvesicular and macrovesicular steatosis. Hepatic triglyceride levels were elevated to a greater degree in male HFD PN + HFD offspring compared with Ctrl PN + HFD male offspring (**Figure 4C**). There was also an increased percentage of collagen fibers stained with picro-sirius red per high-powered field in male HFD PN + HFD offspring, indicating increased liver fibrosis in this group (**Figure 4D**). Taken together, our results show that maternal HFD feeding during lactation increases susceptibility of male mice to hepatic fibrosis and fat accumulation upon HFD rechallenge later in life.

Hepatic Upregulation of Gluconeogenesis, the PPAR-Alpha Pathway, and Inflammation Genes

To further understand the molecular events underlying the liver pathology observed in offspring, we extracted RNA from 6-month adult male offspring liver in a non-fasted state and performed an RNA array using an Affymetrix platform to examine differences in transcriptomes between the Ctrl PN and

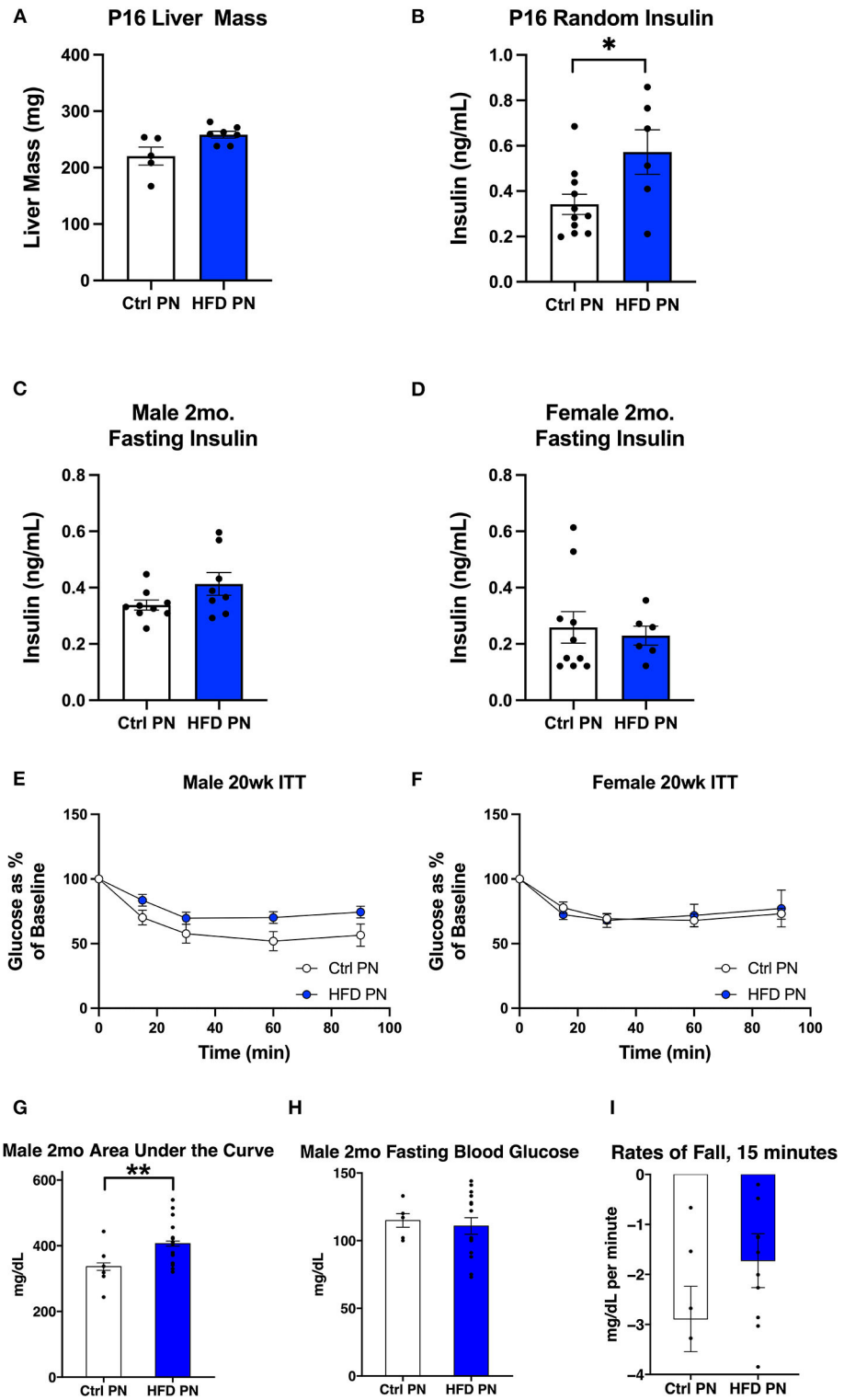

FIGURE 2 | Offspring metabolic characteristics after lactational exposures. All data are presented as a litter average with the n reflecting the number of litters studied. All white values are for Ctrl PN and blue are for HFD PN offspring. **(A)** Post-natal day 16 (P16) Liver Mass **(B)** P16 Fed Insulin **(C)**. Fasting insulin at 2 months in male offspring **(D)**. Fasting insulin at 2 months in female offspring **(E)**. Insulin tolerance test at 5 months in male offspring ($n = 6$–7 litters) **(F)**. Insulin tolerance test at 5 months in female offspring ($n = 4$–7 litters) **(G–I)** constitute further analysis of the 5-month male ITT **(G)**. The area under the curve from 100% for the ITT glucose values **(H)**. Fasting blood glucose at the start of the ITT **(I)**. The rate of fall in blood glucose from the baseline to 15 min during the test. Ctrl, control; HFD, high-fat diet; PN, post-natal. *$p < 0.05$.

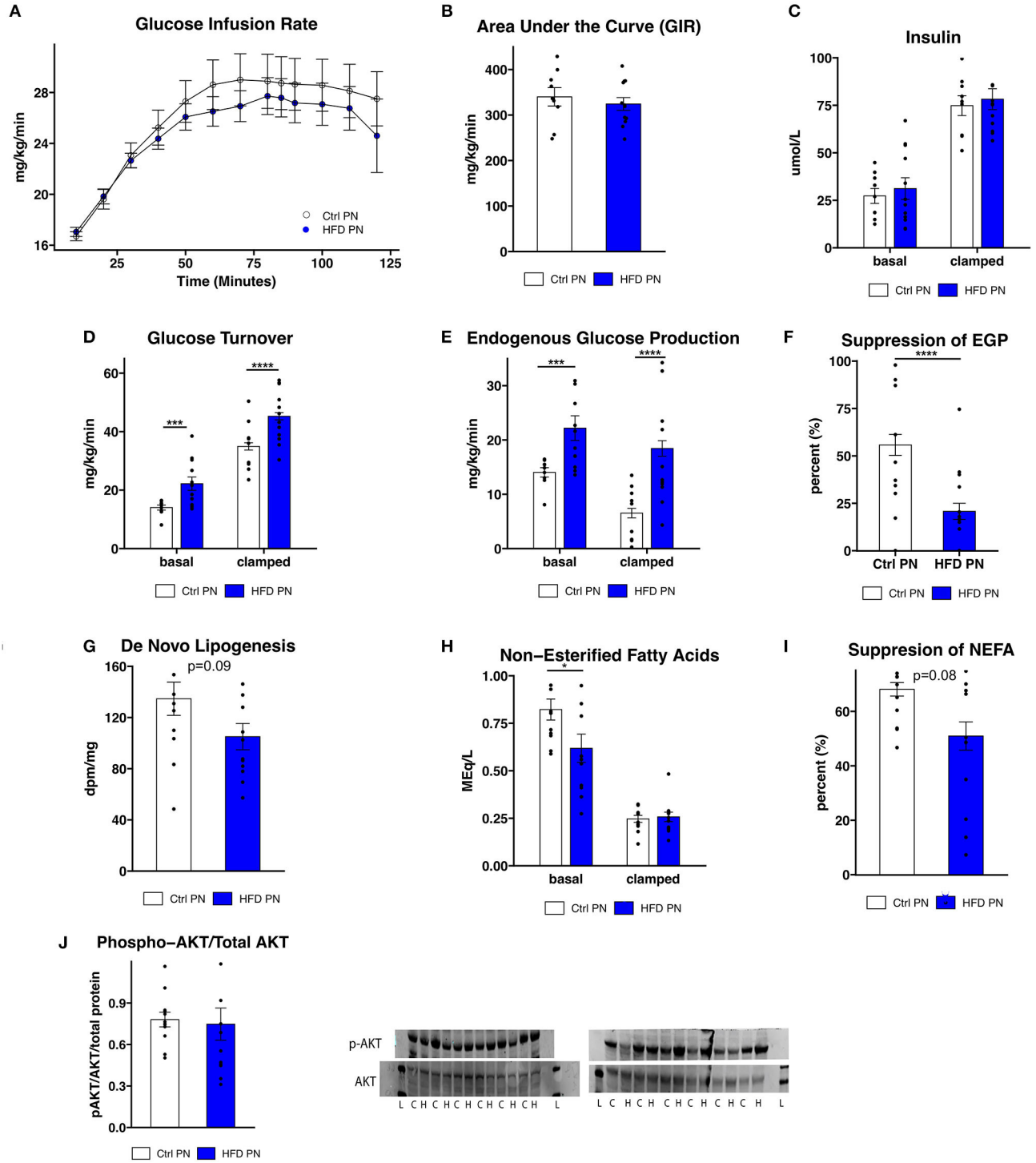

FIGURE 3 | Insulin sensitivity and glucose production. Three-month-old male mice whose mothers either had normal diet (Ctrl PN, $n = 9$) or high-fat diet (HFD PN, $n = 12$) during the lactation period underwent a hyperinsulinemic euglycemic clamp, beginning after a 6-h fast. **(A)** Time course of glucose infusion rate (GIR) over the course of the clamp study. **(B)** The area under the curve for GIR **(C)**. Insulin levels at basal and clamped conditions **(D)**. Glucose turnover at basal and clamped conditions. **(E)** Endogenous glucose production (EGP) at basal and clamped conditions **(F)**. Percent suppression of EGP **(G)**. *De novo* lipogenesis in liver tissue in response to insulin **(H)**. Non-esterified fatty acids (NEFA) in serum at basal and clamped conditions **(I)**. Suppression of NEFA in serum **(J)**. Western blot of liver tissue showing AKT and p-AKT (ser473) protein levels. Western blot abbreviations: C, Ctrl PN; H, HFD PN; L, Ladder. Other abbreviations: Ctrl, control; HFD, high-fat diet; PN, post-natal. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

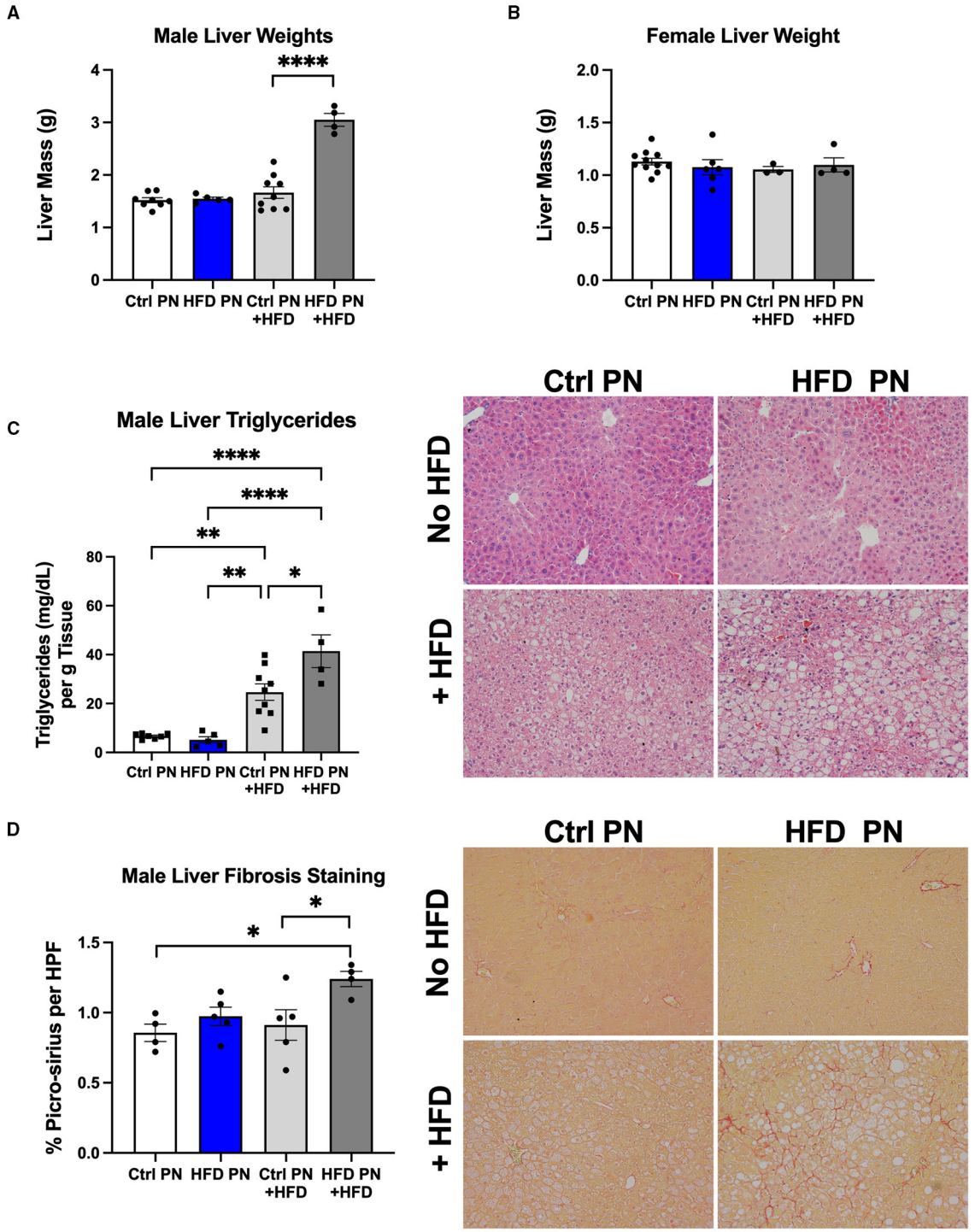

FIGURE 4 | Offspring liver morphology and metabolic characteristics. Liver weights at necropsy (6 months of age) in male **(A)** and female **(B)** offspring during both ND feeding (no HFD) and HFD rechallenge. ND results are white for Ctrl PN and blue for HFD PN. HFD rechallenge results are in light gray for Ctrl PN + HFD and dark gray for HFD PN + HFD **(C)**. Liver triglyceride quantification and representative histology by hematoxylin and eosin (H&E) staining to visualize fat accumulation as open spaces. **(D)** Liver fibrosis quantification by picro-sirius staining with representative images showing red-stained collagen fibers. Ctrl, control; HFD, high-fat diet; PN, post-natal; HPF, high-power field. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

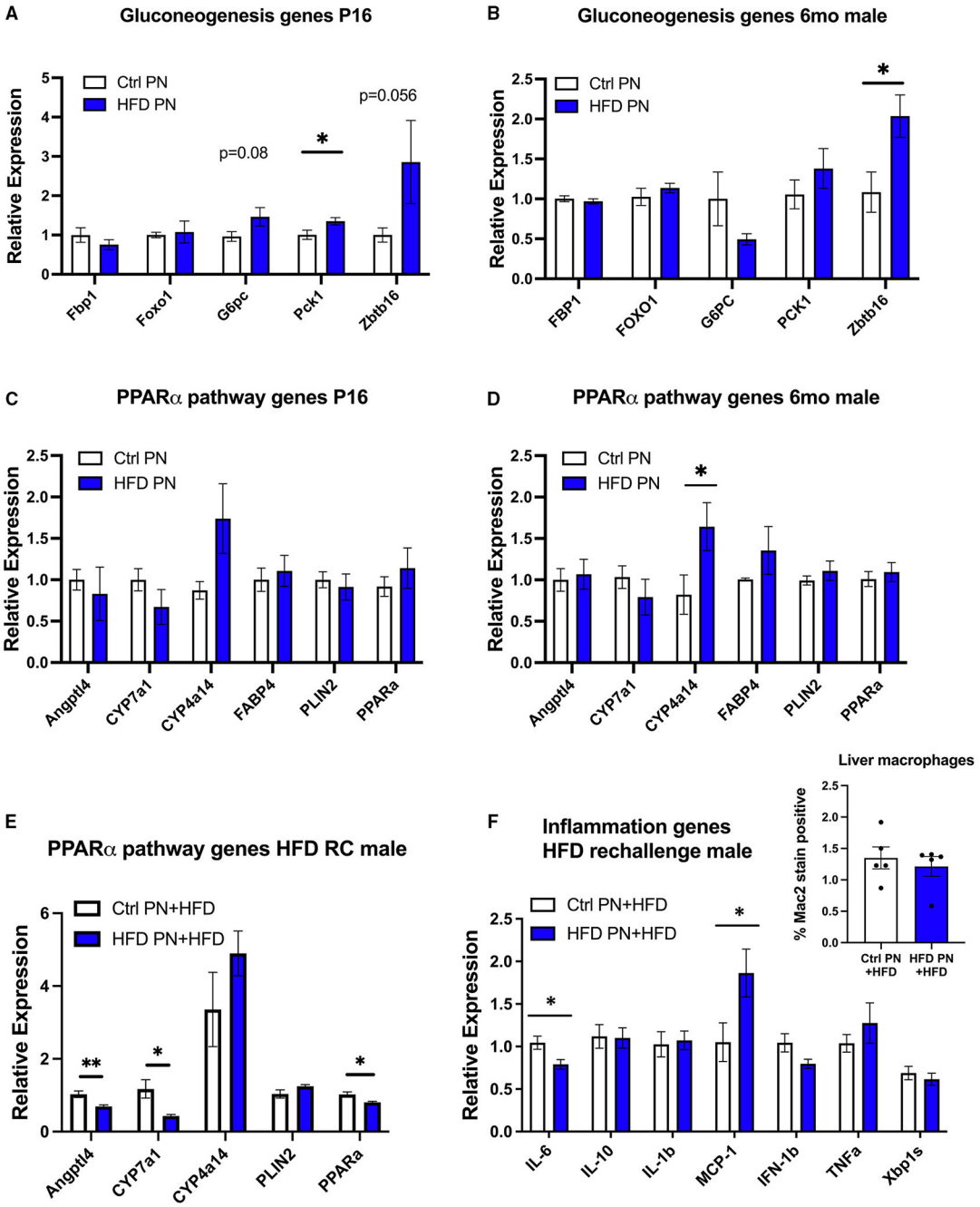

FIGURE 5 | Offspring hepatic gene expression across the lifespan. Gene expression data are representative of individual mice and not averaged per litter. White values are for Ctrl PN, and blue are for HFD PN offspring. Gluconeogenesis genes at post-natal day 16 (P16) Ctrl PN $n = 9$–10, HFD PN $n = 4$–7 per gene **(A)** and in adult male offspring maintained on ND at 6-month Ctrl PN $n = 4$–5, HFD PN = 4–5 per gene **(B)**. PPAR-alpha pathway genes at P16 Ctrl PN $n = 9$–12, HFD PN $n =$ 10–13 per gene **(C)**, and in adult male offspring maintained on ND at 6-month Ctrl PN $n = 7$–8, HFD PN $n = 7$–9 per gene **(D,E)**. PPAR-alpha pathway genes in male offspring rechallenged with HFD at 6-months Ctrl PN + HFD $n = 8$, HFD PN + HFD $n = 8$ per gene **(F)**. Inflammatory genes in male offspring rechallenged with HFD at 6-months Ctrl PN + HFD $n = 6$–8, HFD PN + HFD $n = 8$ per gene with quantitation of liver macrophages in HFD rechallenged males at 6 months using immunofluorescent staining for Mac2 (inset). Ctrl, control; HFD, high-fat diet; PN, post-natal. $^*p < 0.05$, $^{**}p < 0.01$.

HFD PN groups. When using an exploratory p-value cut-off of $p = 0.01$, there were 85 genes with increased expression and 18 genes with decreased expression in HFD PN males compared with Ctrl PN. When the results of this pilot gene expression study were analyzed using gene set enrichment analysis, the major enhanced pathway was the peroxisome proliferator-activated

receptor (PPAR)-alpha pathway. Among the genes with the most elevated expression in HFD PN males were *Cyp4a14* and *Zbtb16*. Among those with the highest effect size for decreased relative expression was *Cyp7a1*. We then went on to perform individual qPCR on a larger set of samples to validate the array results and to examine similar gene candidates at other time points during the life course.

Due to the changes in hepatic glucose production, we first analyzed genes related to hepatic gluconeogenesis. At P16, we detected a significant increase in the gluconeogenesis enzyme *Pck1* with a trend toward an increase in *G6pc* and *Zbtb16*, although this did not reach statistical significance ($p = 0.08$ and $p = 0.056$, respectively, **Figure 5A**). In adult male HFD PN liver, only *Zbtb16* had significantly increased expression (**Figure 5B**). Given the significant enrichment of PPAR-alpha pathway genes in our pilot array, we examined these candidates in P16 liver where there were no differences between groups that reached statistical significance (**Figure 5C**). In the adult male HFD PN offspring, we detected a significant increase in *Cyp4a14* (**Figure 5D**). In adult male HFD PN + HFD offspring, we detected some different changes in the PPAR-alpha pathway with decreased *Angptl4*, *Cyp7a1*, and *Ppara* (**Figure 5E**). Overall, *Cyp4a14* was highly expressed in both HFD rechallenge groups. We previously showed an increase of inflammatory macrophages in gonadal white adipose tissue in HFD PN + HFD male mice (8). Therefore, we examined expression of inflammatory genes and found elevated *Mcp-1* and decreased *Il-6* expression in HFD PN + HFD male livers (**Figure 5F**). When we examined the livers with Mac2 immunofluorescent staining to evaluate for the presence of macrophages, there was no clear difference between the Ctrl PN + HFD and HFD PN + HFD groups (**Figure 5F**, inset). Overall, we found an increase in *Zbtb16* and *Cyp4a14* expression in adult males that may be associated with lactational HFD exposure and could explain liver phenotypes detected in our model.

Maternal Metformin Administration in Addition to HFD Counteracts Some Offspring Metabolic Outcomes

We have previously demonstrated the ability of metformin exposure during lactation to program decreased offspring adiposity and protection from the effects of HFD rechallenge (20). We next wanted to understand the impact of metformin given to dams at the same time as lactational HFD on offspring metabolic outcomes. We observed that metformin returned the suckling offspring weights on days 7, 14, and 21 to the same as those of the Ctrl PN offspring (**Figure 6A**). HFD + Met PN offspring had lower-fasting insulin levels at 2 months of age than both Ctrl PN and HFD PN offspring (**Figure 6B**), and had decreased visceral adiposity at 6 months of age, as measured by GWAT weight (**Figure 6C**), compared to HFD PN males. During the 5-month ITT, the HFD + Met PN males were more responsive to insulin administration than the HFD PN males. However, HFD + Met PN males did not fully recapitulate the insulin sensitivity of Ctrl PN males (**Figure 6D**). Under hyperinsulinemic euglycemic clamp conditions, the suppression of hepatic glucose production of HFD + Met PN males did not differ from that of Ctrl PN or HFD PN groups (**Figures 6E,F**). Baseline NEFA levels tended to normalize in HFD + MetPN offspring with levels 35% higher than in HFD PN offspring ($p = 0.078$). Suppression of lipolysis also tended to improve in the HFD + MetPN group with suppression 35% higher than in HFD PN, although this did not reach significance ($p = 0.10$, **Figure 6G**). In terms of the key liver genes altered by lactational HFD, metformin increased expression of *Ppara* along with decreasing *Plin2* and *Zbtb16* (**Figure 6H**).

DISCUSSION

In these experiments, we demonstrated that a brief exposure of dams to HFD during lactation has lifelong implications for the offspring and that concurrent maternal metformin exposure may provide some protection in adulthood. Metabolic abnormalities were detected in HFD PN offspring on the normal diet, which included liver and adipose tissue insulin resistance. A "second-hit" stressor of HFD in adult life caused progression to liver steatosis to a greater extent in HFD PN offspring, and fibrosis which was not observed in Ctrl PN offspring. When metformin was given to dams being fed HFD, some of the metabolic phenotypes in male offspring were rescued along with a lower-fasted insulin level and a trend toward improved liver sensitivity to insulin. There are some reports that elevated insulin levels/hyperinsulinemia alone can precipitate insulin resistance and obesity, thus making the lower insulin levels in the Met + HFD PN of potential benefit (33, 34).

In the setting of the hyperinsulinemic euglycemic clamp, we found evidence of hepatic, cardiac, and adipose insulin resistance, including impaired suppression of lipolysis and glucose production in male HFD PN offspring. In this experiment, there was no difference in glucose infusion rates, indicating that increased hepatic glucose output in the HFD PN male offspring could be offset by increased peripheral glucose turnover. However, there was no evidence of increased glucose turnover in the tissues assessed here. It is possible that this increased glucose turnover is occurring in a tissue that was not analyzed during the clamp, for example, the brain. The increased whole-body glucose turnover rate at the baseline prior to insulin stimulation would also support this possibility, as brain glucose uptake is largely insulin independent (35). Other groups have shown the brain to be highly susceptible to programming in response to maternal HFD (32, 36). In a porcine model with high-fat diet given in the preconception period through lactation offspring had increased brain glucose uptake as newborns (37). However, this subsided at 1 month of age. The main effect of the metformin co-treatment seems to have been on the liver with no evidence of rescue of the muscle or adipose insulin resistance. However, in the Met + HFD PN group, clamp suppression of lipolysis became similar to Ctrl PN. Metformin is detected at higher levels in the portal circulation, making a liver-predominant effect in the offspring possible, despite having very low circulating pup metformin levels in our other lactational metformin models (20).

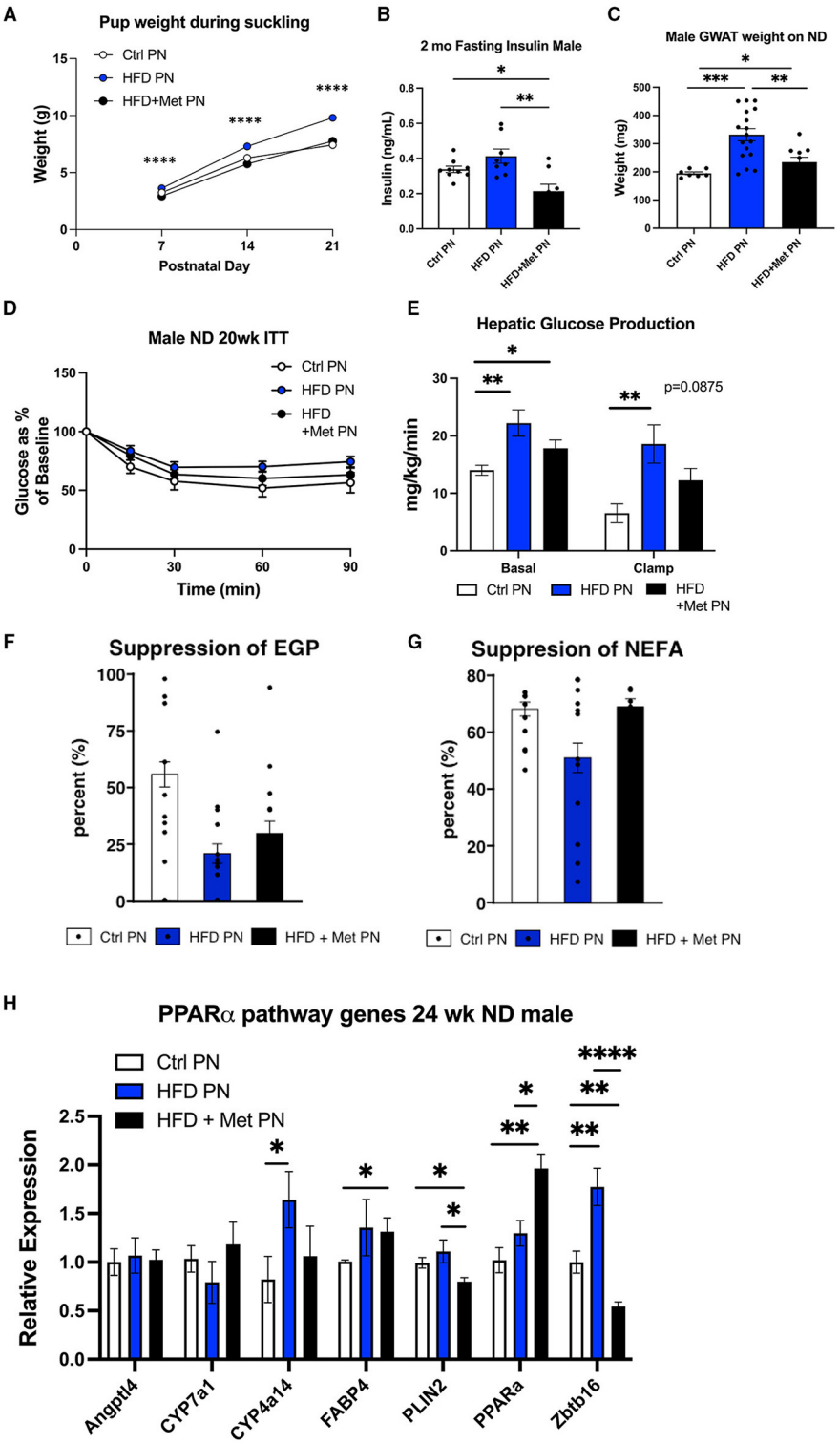

FIGURE 6 | Impact of metformin given during lactation at the time of HFD exposure on offspring. White values are for Ctrl PN, blue are for HFD PN, and black are for HFD + Met PN offspring **(A)**. Pup weights during lactation averaged by litter ($n = 6$–7 litters) **(B)**. Fasting insulin in male offspring at 2 months of age **(C)**. Visceral fat pad weight (GWAT) in male offspring at necropsy at 6 months **(D)**. Insulin tolerance test at 5 months ($n = 7$–8) **(E)**. Hepatic glucose production at basal and clamped conditions during the hyperinsulin mice uglycemic clamp (HEC). This is the same clamp as in **Figure 3** in which Ctrl PN and HFD PN results were shown, repeated here with the addition of the HFD + Met PN group ($n = 8$–11) **(F)**. Percent suppression of endogenous glucose production **(G)**. Percent suppression of lipolysis on clamp **(H)**. PPAR-alpha pathway genes in adult male offspring-fed ND at 6 months ($n = 5$–8). Ctrl PN and HFDPN expression results were previously shown in **Figure 5D**, repeated here with the addition of the HFD + Met PN group. Ctrl, control; HFD, high-fat diet; PN, post-natal; Met, metformin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Our pilot gene set enrichment analysis identified upregulated PPAR-alpha pathway signaling in the livers of HFD PN male offspring fed ND. PPAR-alpha pathway responses are thought to be beneficial for the liver. Previous studies have shown that whole body and hepatic *Ppara* knockout animals are prone to developing hepatic steatosis (38). Indeed, PPAR-alpha agonists are being investigated as therapeutic options in NAFLD and metabolic syndrome. There could be two explanations for the PPAR-alpha findings in this study. First, this could represent a stress response that would be beneficial and may be protective for the HFD PN offspring livers. Further studies would be needed to identify the effects of antagonizing the PPAR-alpha in this experimental system. Another explanation is that the members of the PPAR-alpha pathway we found to be altered are not the major contributing members, and that these genes are exerting their effects on the offspring liver independently of PPAR-alpha signaling. Other studies have linked increased *Cpt1a*, *Pdk4*, and *Fgf21* with resolution of NASH in human liver biopsies (39), and we did not detect changes in these targets on the initial transcriptomics study. This study also showed that increased *Ppara* expression associated with NASH resolution, which could be pertinent to the findings in our HFD + MetPN group where liver *Ppara* expression was increased.

Our studies revealed an upregulation of Promyelocytic leukemia zinc finger (PLZF), also known as *Zbtb16*, in male HFD PN mice during the lactational exposure, at P16 and into adulthood. Increased *Zbtb16* gene expression has been observed in multiple models of diabetes, including db/db, streptozotocin (STZ) treated, and HFD-fed mice. Adenovirus-mediated overexpression of *Zbtb16* led to increased hepatic gluconeogenic gene expression, hyperinsulinemia, and insulin resistance in mice (40). In our model, it is possible that changes in the level of *Zbtb16* contributed to the increased hepatic glucose production and insulin resistance phenotypes in HFD PN offspring. *Zbtb16* expression was reduced in livers of HFD + Met PN males. This may represent a potential rescue mechanism provided by lactational metformin exposure, given that we also observed a tendency to improve hepatic glucose production in HFD + Met PN offspring. Further studies are needed to determine if post-natal metformin exposure reduces *Zbtb16* expression in HFD PN male offspring subjected to HFD rechallenge.

Cytochrome P450 omega-hydroxylase 4A14 (*Cyp4a14*) was also upregulated in the livers of HFD PN male mice. *Cyp4a14* is a direct transcriptional target of PPARa and is involved in fatty acid metabolism. Cyp4a family members metabolize arachidonic acid to generate pro-inflammatory mediator 20-hydroxyeicosatetraenoic acid (20-HETE), which is known to activate NFκB (41). In the context of liver disease, genetic ablation of *Cyp4a14* in mice protects against HFD-induced hepatic steatosis and methionine-choline-deficient (MCD)-diet-induced fibrosis and inflammation (42). Pharmacologic inhibition of Cyp4a family members decreased insulin resistance in HFD-fed mice (43). Dysregulation of *Cyp4a14* may contribute to the increased hepatic steatosis in HFD PN male offspring observed in the current study and to previously reported insulin resistance in HFD rechallenged HFD PN male mice (8).

In the pilot transcriptomics data and the "second-hit" male livers, we detected decreased levels of Cytochrome P450 Family 7 Subfamily A Member 1 (Cyp7a1). *Cyp7a1* encodes the rate-limiting enzyme that catalyzes the conversion of cholesterol to 7α-hydroxycholesterol in the classic bile acid synthesis pathway, playing a critical role in cholesterol and bile acid homeostasis (44). It has been shown that *Cyp7a1*$^{-/-}$ mice fed a MCD diet to cause NAFLD have increased hepatic-free cholesterol compared to wild-type mice (45). Free cholesterol may accumulate in hepatic stellate cells and exacerbate liver fibrosis in NASH (46). Additionally, adenovirus-mediated *Cyp7a1* gene transduction has been shown to inhibit lipopolysaccharide (LPS)-induced p65 binding to NF-κB binding sites on cytokine gene promoters, possibly mediated by the activation of FXR via bile acids (45).

The enhanced hepatic steatosis and fibrosis observed in HFD PN + HFD male offspring were accompanied by an increased expression of *Mcp-1* and a decreased expression of *Il-6*. An increase in *Mcp-1* expression would suggest enhanced macrophage infiltration into the liver. However, when liver sections were analyzed for the presence of Mac2^{+} macrophages, we did not observe any difference in infiltration between Ctrl PN + HFD and HFD PN + HFD groups. This could be better assessed using flow cytometry. Within the liver, Kupffer cells and hepatic stellate cells also express the MCP-1 receptor, *Ccr2*. Expression of CCR2 on these cells may also contribute to the development of fibrosis after liver injury; however, this was not shown to be dependent on MCP-1(47). IL-6 is known to play both pro-inflammatory and anti-inflammatory roles in different contexts. In the liver, antibody blockade of IL-6 signaling led to enhanced hepatic steatosis in response to MCD diet (48). Conversely, chronic treatment of ob/ob mice with subcutaneous injection of recombinant IL-6 reduced hepatic steatosis and triglyceride levels (49). Taken together, the expression patterns that we observed in HFD PN + HFD male livers may be contributing pathological changes.

The effects we detected on the liver and insulin sensitivity are sexually dimorphic with the male offspring being predominantly impacted and the female offspring relatively spared. We previously reported the lack of effect of lactational HFD on female offspring insulin sensitivity and adiposity detected in our studies out to age 6 months (8). Male sex confers an increased risk of NAFLD in a clinical setting, and, in our mouse experimental results, we also detected this difference in outcomes. While both male and female offspring are heavier as a result of lactational HFD during the suckling period, when we study the offspring as young adults, there were no evident metabolic phenotypes in females. Perhaps, the effect of pubertal hormone exposure is protective in females. Indeed, *Cyp4a14* has sexually dimorphic expression in mice with increased levels in females. The decreased expression in males was caused by male-specific growth hormone secretory patterns and androgens (50). This may indicate a different role for this enzyme in female mice or different sensitivity to elevated levels in males. Finally, it is possible that the female mice have a milder phenotype that may be unmasked by a longer HFD rechallenge period or the addition of a stressor, like pregnancy.

In summary, these studies have provided evidence of a complex offspring response to lactational HFD exposures that leads to hepatic insulin resistance and increased risk of NAFLD in adult male offspring. Male HFD PN offspring also have evidence of insulin resistance in the heart and adipose tissue. We have identified potential molecular candidates that could be associated with the liver abnormalities observed in this model as they have been shown to cause insulin resistance, increased gluconeogenesis, hepatic steatosis, and fibrosis in other models. The unique aspects of this study are that a brief, indirect neonatal exposure to HFD may contribute to genetic perturbations in adulthood, leading to lifelong metabolic disease risk. Future studies will be designed to understand the impacts of these specific genes on the liver across the life span and how changes in the maternal milk that results from HFD exposure can regulate expression of these genes, possibly through epigenetic changes. Finally, we will also determine if the co-exposure of dams to HFD and metformin during lactation is able to rescue the offspring from the detrimental effects of an HFD "second-hit" stressor.

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/geo/query/acc.cgi?acc=GSE182071.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Michigan Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

BG was responsible for the study design and conceived the original idea. BG, HH, MM, ZC, HS, MW, and NQ carried out the experiments. BG, HH, MM, ZC, NQ, and DB performed data analysis. Manuscript preparation was done by BG, HH, MM, ZC, PH, HS, and DB. Manuscript editing was completed by HH, MM, ZC, NQ, DB, and BG. Input and approval of the final manuscript were provided by all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.759690/full#supplementary-material

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Maternal Pre-pregnancy Body Mass Index Categories and Infant Birth Outcomes: A Population-Based Study of 9 Million Mother–Infant Pairs

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Background and Aims: Infant adverse birth outcomes have been suggested to contribute to neonatal morbidity and mortality and may cause long-term health consequences. Although evidence suggests maternal prepregnancy body mass index (BMI) categories associate with some birth outcomes, there is no consensus on these associations. We aimed to examine the associations of maternal prepregnancy BMI categories with a wide range of adverse birth outcomes.

Methods: Data were from a population-based retrospective cohort study of 9,282,486 eligible mother–infant pairs in the U.S. between 2016 and 2018. Maternal prepregnancy BMI was classified as: underweight (< 18.5 kg/m^2); normal weight (18.5–24.9 kg/m^2); overweight (25.0–29.9 kg/m^2); obesity grade 1 (30–34.9 kg/m^2); obesity grade 2 (35.0–39.9 kg/m^2); and obesity grade 3 (≥ 40 kg/m^2). A total of six birth outcomes of the newborn included preterm birth, low birthweight, macrosomia, small for gestational age (SGA), large for gestational age (LGA), and low Apgar score (5-min score < 7).

Results: Maternal prepregnancy overweight and obesity increased the likelihood of infant preterm birth, with odds ratios (ORs) (95% CIs) of 1.04 (1.04–1.05) for overweight, 1.18 (1.17–1.19) for obesity grade 1, 1.31 (1.29–1.32) for obesity grade 2, and 1.47 (1.45–1.48) for obesity grade 3, and also for prepregnancy underweight (OR = 1.32, 95% CI = 1.30–1.34) after adjusting for all potential covariates. Prepregnancy overweight and obesity were associated with higher odds of macrosomia, with ORs (95% CIs) of 1.53 (1.52–1.54) for overweight, 1.92 (1.90–1.93) for obesity grade 1, 2.33 (2.31–2.35) for obesity grade 2, and 2.87 (2.84–2.90) for obesity grade 3. Prepregnancy overweight and obesity was associated with higher odds of LGA, with ORs (95% CIs) of 1.58 (1.57–1.59) for overweight, 2.05 (2.03–2.06) for obesity grade 1, 2.54 (2.52–2.56) for obesity grade 2, and 3.17 (3.14–3.21) for obesity grade 3. Prepregnancy overweight and obesity were also associated with higher odds of low Apgar score, with ORs (95% CIs) of

1.12 (1.11–1.14) for overweight, 1.21 (1.19–1.23) for obesity grade 1, 1.34 (1.31–1.36) for obesity grade 2, and 1.55 (1.51–1.58) for obesity grade 3.

Conclusion: Our findings suggest maintaining or obtaining a healthy body weight for prepregnancy women could substantially reduce the likelihood of important infant adverse birth outcomes.

Keywords: pre-pregnancy, body mass index, obesity, preterm birth, low birthweight, macrosomia, small for gestational age, large for gestational age

INTRODUCTION

Prepregnancy well-being of women of reproductive age and their male partners is the basis of healthy pregnancy that contributes to healthy growth and development of the offspring *in utero*. Some adverse birth outcomes such as preterm birth, low birthweight, or macrosomia are important clinical and public health concerns as they increase the incidence of death of the newborns and might lead to long-term health consequences (1–3). Maternal prepregnancy malnutrition is also one of the most common clinical phenomena that may affect birth outcomes of the newborns. It is estimated that global obesity prevalence will surpass 21% and severe obesity will surpass 9% in women by 2025 (4). Among US women, an increasing trend in obesity was found, with the prevalence from 31.5 in 2011 to 2012 to 56.9% in 2017 to 2018 (5). Thus, it is very necessary to explore the associations between maternal prepregnancy body mass index (BMI) and a wide range of adverse birth outcomes.

Maternal prepregnancy overweight and obesity have been associated with several adverse birth outcomes, including preterm birth, macrosomia, and large for gestational (LGA), and prepregnancy underweight is associated with low birthweight and small for gestational age (SGA) (2, 6). However, there is no consensus on these associations. A meta-analysis of 11 studies including 452,991 participants showed no significant association between prepregnancy obesity and preterm birth (odds ratio [OR] = 1.21, 95% CI: 0.95–1.53) and a weak association of prepregnancy underweight with preterm birth (OR = 1.13, 1.01–1.27) (7). Another meta-analysis of 60 studies including 1,392,799 participants reported that prepregnancy obesity had a positive association with low birthweight (OR = 1.24, 1.09–1.41) and a weak association with preterm birth (OR = 1.05, 1.01–1.09) (8). A cohort study among 12,029 pregnant women suggested that prepregnancy obesity increased the risk of LGA and even SGA (9). In addition, although a meta-analysis of 11 studies including 2,586,265 participants suggested maternal overweight and obesity were associated with low Apgar score (10), authors of a subsequent population-based cohort (N= ∼2,000) concluded there was no difference in the odds of low Apgar score among offspring of women with normal weight, overweight and obesity before pregnancy (11). Thus, the inconsistent associations of maternal prepregnancy BMI categories with a wide range of adverse birth outcomes require confirmation.

In this study, we examined the associations of maternal prepregnancy BMI categories (from underweight to obesity grade 3) with a wide range of infant adverse birth outcomes (i.e., preterm birth, low birthweight, macrosomia, SGA, LGA, and low Apgar score) in a population-based national study of 9 million mother–infant pairs.

MATERIALS AND METHODS

Data Source

We used national birth certificate data from the U.S. National Vital Statistics System (NVSS), which is a retrospective, population-based cohort study that includes information on a wide range of maternal and infant demographics and health characteristics for all births occurring in 50 states and the District of Columbia in the U.S. Birth certificate data from each registration area are received by the Vital Statistics Cooperative Program, the National Center for Health Statistics of the Centers for Disease Control and Prevention. The NVSS data collection methodology, quality control, and vital statistics are publicly available on the CDC website (https://www.cdc.gov/nchs/nvss/births.htm).

In this study, we used 2016–2018 NVSS data because all the US states and the District of Columbia had completely implemented the 2003 version of the Standard Certificate of Live Birth to collect birth information since 2016. We initially extracted all live births of 11,622,400 mother–infant pairs between 2016 and 2018 in the U.S. with 3,956,112 in 2016, 3,864,754 in 2017, and 3,801,534 in 2018. We excluded 549,640 because of age of women < 18 or ≥ 50 years, or twin or multiple births, 276,906 because of missing data on maternal prepregnancy BMI, or any infant outcomes (gestational age, birthweight, and 5-min Apgar score), and 1,513,368 because o women with prepregnancy hypertension or diabetes, or missing data on maternal characteristics, maternal smoking status during pregnancy, or pregnancy history or prenatal care. Finally, a total of 9,282,486 eligible mother–infant pairs were included for this analysis. **Figure 1** provides a flow chart of inclusion/exclusion of mother–infant participants from the 2016 to 2018 birth certificate data in NVSS.

Data Collection

Maternal age at delivery was calculated from the date of birth of the mother. Maternal prepregnancy BMI was calculated as prepregnancy weight in kilograms divided by the square of height in meters. Maternal height and weight were self-reported by the women, and weight was maternal weight immediately before the woman became pregnant with the child included in this study. Maternal race/ethnicity was divided into Hispanic, non-Hispanic white, non-Hispanic black, and others. Maternal

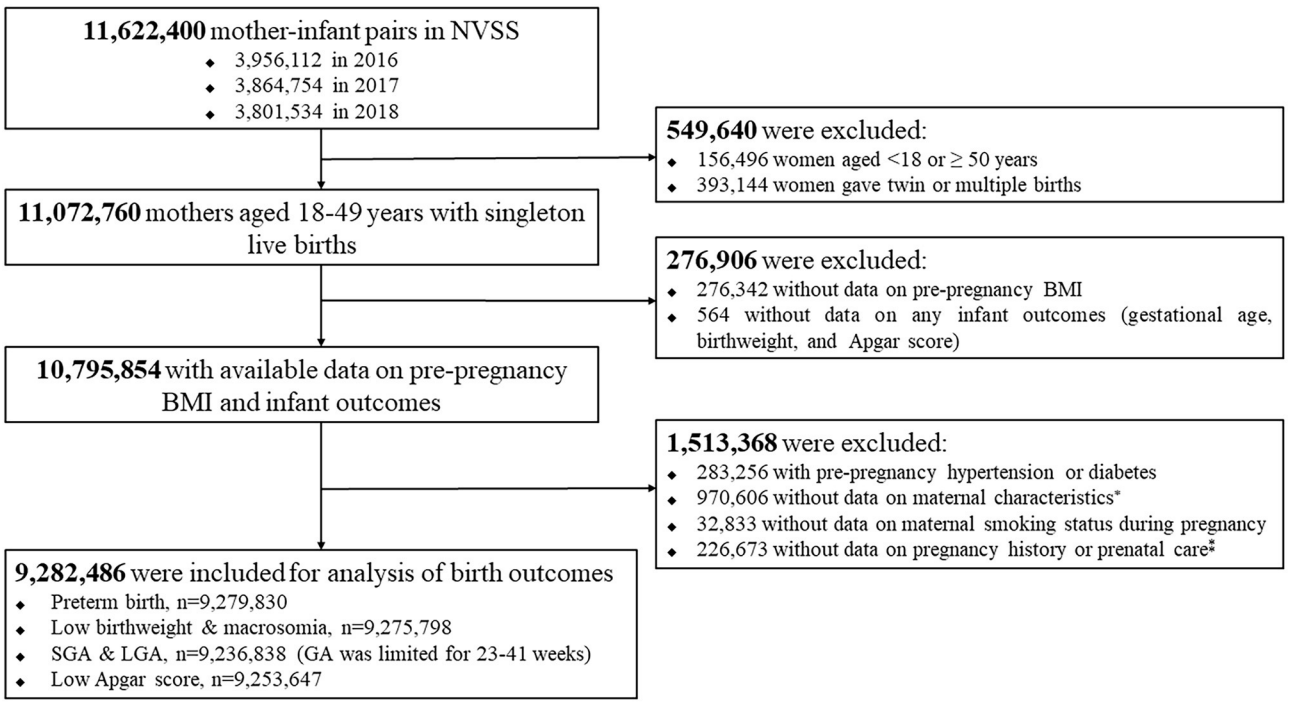

FIGURE 1 | Flow chart of inclusion/exclusion of the study population. *maternal age at delivery, race/ethnicity, education level, marital status; ‡The number of live births, and the total number of prenatal care visits.

education levels were collected as the highest level of education at the time of delivery. Marital status was reported as "yes" or "no." Smoking status during pregnancy was collected as "yes" or "no" during pregnancy. Eclampsia, gestational hypertension, and diabetes, separately, were diagnosed during pregnancy as "yes" and "no." Live-birth order indicated what number the present birth represents; for example, if a baby is born to a mother who has had two previous live births, this baby will have a live-birth order of the third. A total number of prenatal care visits for this pregnancy was also collected. The infant sex was categorized as male and female. Gestational age was calculated based on the obstetric estimate of gestation at delivery. Infant birth weight was reported in grams, and if the weight in grams was not available, weight in pounds or ounces was converted to grams. Apgar score indicated a systematic measure for evaluating the physical condition of an infant at specific intervals at birth, and a 5-min score was recorded from 0 to 10. Additional information on these variables can be obtained from the User Guides to the 2016–2018 Natality Public Use Files (12–14).

Definitions of Maternal Prepregnancy BMI Categories and Related Covariates

Maternal prepregnancy BMI was classified as: underweight (< 18.5 kg/m^2); normal weight (18.5–24.9 kg/m^2); overweight (25.0–29.9 kg/m^2); obesity grade 1 (30–34.9 kg/m^2); obesity grade 2 (35.0–39.9 kg/m^2); and obesity grade 3 (≥ 40 kg/m^2). We categorized related covariates as follows: maternal age at delivery as < 30 years, and ≥ 30 years, maternal race/ethnicity as Hispanic, non-Hispanic white, non-Hispanic black, and other, maternal education level as less than high school, high school, and more than high school, marital status as married and unmarried, smoking status during pregnancy as yes and no, live-birth order as 1, 2, and ≥ 3, infant sex as male and female, total number of prenatal care visits as 0, 1–4, 5–9, and ≥ 10, eclampsia as yes and no, gestational hypertension as yes and no, and gestational diabetes as yes and no.

Definitions of Birth Outcomes

We included six adverse birth outcomes: preterm birth, low birthweight, macrosomia, SGA, LGA, and low Apgar score. Preterm birth was defined as gestational age at delivery < 37 weeks. Low birthweight was defined as birth weight $< 2{,}500$ g, and macrosomia as birth weight $\geq 4{,}000$ g. SGA and LGA were defined as birth weight below the 10th percentile values (for SGA), and above the 90th percentile values (for LGA) by gestational age and sex according to the U.S. new intrauterine growth curves (15). We defined low Apgar score as a 5-min score less than 7 that indicated an infant in the intermediate or less physical condition.

Statistical Analysis

Population characteristics were presented using median (interquartile range, IQR) for continuous variables and *n* (%) for categorical variables. Binary or multinomial logistic regression models were used to calculate ORs with 95% CIs of adverse birth outcomes (i.e., preterm birth, low birthweight,

macrosomia, SGA, LGA, and low Apgar score) according to maternal prepregnancy underweight, overweight, obesity grade 1, obesity grade 2, and obesity grade 3 relative to the reference group of normal weight. To address the impact of potential confounders, we performed three logistic regression models: Model 1 was unadjusted model; Model 2 was the simpler model adjusted for two demographic factors including maternal age at delivery and race/ethnicity; Model 3 was the model adjusted for more potential confounders to show the more reliable results, including adjustment for maternal age at delivery, race/ethnicity, education levels, marital status, smoking status during pregnancy, live-birth order, infant sex, gestational age (for low birthweight, macrosomia, or low Apgar score only), and a total number of prenatal care visits. We considered the included confounders based on the available variables from the original NVSS data and also from previous similar studies (16, 17). Subgroup analyses were performed by maternal race/ethnicity, maternal age at delivery, and infant birth year. To assess the stability of our findings, two sensitivity analyses were performed by excluding women with cesarean section, and by excluding those with eclampsia, gestational hypertension, or diabetes. We also assessed the dose–response relationship between prepregnancy BMI (as a continuous variable) and infant adverse birth outcomes using restricted cubic spline (RCS) logistic regression models with adjustment for all potential covariates, with three knots of the 5, 50,' and 95th percentiles of the distribution of the continuous prepregnancy BMI (18). RCS_Reg macro was employed for the RCS analysis under SAS 9.4 software. Data cleaning and all analyses were performed by SAS 9.4 (SAS Institute Inc., Cary, North Carolina). A two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the Study Population

Table 1 presents characteristics of the study population by maternal prepregnancy BMI category. Among 9,282,486 pregnant women, prepregnancy BMI was divided into underweight (311,445, 3.4%), normal weight (4,067,646, 43.8%), overweight (2,451,775, 26.4%), obesity grade 1 (1,351,922, 14.6%), obesity grade 2 (652,581, 7.0%), and obesity grade 3 (447,117, 4.8%). Overall maternal age at delivery was 29 (IQR: 25–33) years, with 54.9% for $<$ 30 years and 45.1% for $\geq$ 30 years. Overall race/ethnicity consisted of Hispanic (21.5%), non-Hispanic white (55.1%), non-Hispanic black (14.5%), and others (8.9%). Compared with women with prepregnancy normal weight, those with prepregnancy underweight or obesity tended to have lower education levels, be unmarried, and smoke cigarettes during pregnancy.

Associations of Maternal Prepregnancy BMI Categories With Infant Birth Outcomes

Preterm Birth

In the fully adjusted model, maternal prepregnancy underweight was associated with higher odds of preterm birth (OR = 1.32, 95% CI = 1.30–1.34); prepregnancy overweight and obesity also increased the odds of preterm birth, with ORs (95% CIs) of 1.04 (1.04–1.05) for overweight, 1.18 (1.17–1.19) for obesity grade 1, 1.31 (1.29–1.32) for obesity grade 2, and 1.47 (1.45–1.48) for obesity grade 3 (**Table 2**).

Low Birthweight and Macrosomia

In the fully adjusted model, maternal prepregnancy underweight was associated with increased odds of low birthweight, with OR (95% CI) of 1.64 (1.61–1.66), and prepregnancy overweight, and obesity were also associated with higher odds of macrosomia, with ORs (95% CIs) of 1.53 (1.52–1.54) for overweight, 1.92 (1.90–1.93) for obesity grade 1, 2.33 (2.31–2.35) for obesity grade 2, and 2.87 (2.84–2.90) for obesity grade 3 (**Table 2**).

Small for Gestational Age and Large for Gestational Age

In the fully adjusted model, maternal prepregnancy underweight was associated with higher odds of SGA, with OR (95% CI) of 1.56 (1.54–1.58), and prepregnancy overweight and obesity were associated with higher odds of LGA, with ORs (95% CIs) of 1.58 (1.57–1.59) for overweight, 2.05 (2.03–2.06) for obesity grade 1, 2.54 (2.52–2.56) for obesity grade 2, and 3.17 (3.14–3.21) for obesity grade 3 (**Table 2**).

Low Apgar Score

In the fully adjusted model, maternal prepregnancy overweight and obesity were associated with higher odds of low Apgar score, with ORs (95% CIs) of 1.12 (1.11–1.14) for overweight, 1.21 (1.19–1.23) for obesity grade 1, 1.34 (1.31–1.36) for obesity grade 2, and 1.55 (1.51–1.58) for obesity grade 3 (**Table 2**).

Subgroup and Sensitivity Analyses of Maternal Prepregnancy BMI Categories With Infant Birth Outcomes

Subgroup analyses by maternal race/ethnicity, maternal age at delivery, and infant birth year showed largely similar results to those shown for the main analyses (**Supplementary Table S1**).

Two sensitivity analyses (exclusion of women with cesarean section, exclusion of those with eclampsia, gestational hypertension or diabetes in models) confirmed the consistency of our findings (**Supplementary Table S2**).

Dose–Response Relationships of Maternal Pre-pregnancy BMI With Infant Birth Outcomes

As shown in **Figure 2**, either a higher or lower maternal prepregnancy BMI increased the odds of preterm birth; a higher maternal prepregnancy BMI was associated with higher odds of macrosomia, LGA and low Apgar score, and a lower maternal prepregnancy BMI was associated with higher odds of low birthweight and SGA. Of note, women with a prepregnancy normal BMI range of 18.5 to 25.0 kg/m^2 in this

TABLE 1 | Characteristics of the study population by maternal prepregnancy BMI categories.

Characteristics	Total	Underweight	Normal weight	Overweight	Obesity grade 1	Obesity grade 2	Obesity grade 3
N	9,282,486	311,445	4,067,646	2,451,775	1,351,922	652,581	447,117
Pre-pregnancy BMI, kg/m^2, median (IQR)	25.4(22.1–30.2)	17.7(17–18.1)	22.1(20.7–23.5)	27.1(25.8–28.3)	32.0(30.9–33.3)	37.0(35.9–38.3)	43.4(41.5–46.6)
Maternal age at delivery, years							
Median (IQR)	29(25–33)	26(22–31)	29(25–33)	29(25–33)	29(25–33)	28(25–33)	28(25–33)
Age category, years, n (%)							
<30	5,100,272(54.9)	212,603(68.3)	2,198,628(54.1)	1,311,719(53.5)	750,054(55.5)	371,385(56.9)	255,883(57.2)
≥30	4,182,214(45.1)	98,842(31.7)	1,869,018(45.9)	1,140,056(46.5)	601,868(44.5)	281,196(43.1)	191,234(42.8)
Race/ethnicity, n (%)							
Hispanic	1,999,552(21.5)	50,761(16.3)	761,083(18.7)	606,876(24.8)	344,672(25.5)	148,853(22.8)	87,307(19.5)
Non-Hispanic white	5,112,870(55.1)	168,520(54.1)	2,415,292(59.4)	1,281,733(52.3)	677,208(50.1)	338,348(51.9)	231,769(51.8)
Non-Hispanic black	1,341,257(14.5)	42,231(13.6)	450,686(11.1)	366,026(14.9)	244,148(18.1)	130,976(20.1)	107,190(24.0)
Other	828,807(8.9)	49,933(16.0)	440,585(10.8)	197,140(8.0)	85,894(6.4)	34,404(5.3)	20,851(4.7)
Maternal education level, n (%)							
Less than high school	1,125,417(12.1)	47,532(15.3)	438,582(10.8)	320,222(13.1)	184,185(13.6)	82,119(12.6)	52,777(11.8)
High school	2,384,797(25.7)	96,472(31.0)	932,159(22.9)	624,394(25.5)	387,920(28.7)	199,104(30.5)	144,748(32.4)
More than high school	5,772,272(62.2)	167,441(53.8)	2,696,905(66.3)	1507,159(61.5)	779,817(57.7)	371,358(56.9)	249,592(55.8)
Marital status, n (%)							
Married	5,673,853(61.1)	166,028(53.3)	2,653,552(65.2)	1,497,432(61.1)	766,320(56.7)	357,360(54.8)	233,161(52.1)
Unmarried	3,608,633(38.9)	145,417(46.7)	1,414,094(34.8)	954,343(38.9)	585,602(43.3)	295,221(45.2)	213,956(47.9)
Smoking status during pregnancy, n (%)							
Yes	680,731(7.3)	39,866(12.8)	277,359(6.8)	164,100(6.7)	103,922(7.7)	55,305(8.5)	40,179(9.0)
No	8,601,755(92.7)	271,579(87.2)	3,790,287(93.2)	2,287,675(93.3)	1,248,000(92.3)	597,276(91.5)	406,938(91.0)
Live birth order, n (%)							
0	3,512,701(37.8)	143,684(46.1)	168,8065(41.5)	878,229(35.8)	444,064(32.9)	212,726(32.6)	145,933(32.6)
1	3,020,379(32.5)	96,382(31.0)	1,327,603(32.6)	800,879(32.7)	437,795(32.4)	212,006(32.5)	145,714(32.6)
2	1,598,304(17.2)	43,724(14.0)	640,440(15.7)	442,392(18.0)	259,916(19.2)	125,439(19.2)	86,393(19.3)
≥3	1,151,102(12.4)	27,655(8.9)	411,538(10.1)	330,275(13.5)	210,147(15.5)	102,410(15.7)	69,077(15.5)
Infant sex, n (%)							
Male	4,748,518(51.2)	158,795(51.0)	2,081,128(51.2)	1,255,650(51.2)	691,613(51.2)	333,228(51.1)	228,104(51.0)
Female	4,533,968(48.8)	152,650(49.0)	1,986,518(48.8)	1,196,125(48.8)	660,309(48.8)	319,353(48.9)	219,013(49.0)
Total number of prenatal care visits, n (%)							
0	144,306(1.6)	7,027(2.3)	65,013(1.6)	38,035(1.6)	19,931(1.5)	8,725(1.3)	5,575(1.3)
1–4	343,212(3.7)	15,243(4.9)	146,893(3.6)	91,002(3.7)	50,747(3.8)	23,422(3.6)	15,905(3.6)
5–9	1,927,755(20.8)	73,168(23.5)	841,602(20.7)	511,705(20.9)	281,822(20.9)	131,127(20.1)	88,331(19.8)
≥10	6,867,213(74.0)	216,007(69.4)	3,014,138(74.1)	1,811,033(73.9)	999,422(73.9)	489,307(75.0)	337,306(75.4)

BMI, body mass index; IQR, interquartile range.

observed population was also associated with higher odds of low birthweight and SGA.

DISCUSSION

Our study examined associations of maternal prepregnancy BMI categories with a wide range of adverse birth outcomes, showing that both prepregnancy overweight/obesity and underweight increased the likelihood of preterm birth; whereas prepregnancy overweight and obesity were associated with an increased likelihood of macrosomia, LGA and low Apgar score, and prepregnancy underweight with higher odds of low birthweight and SGA. However, our data did not lend support to prepregnancy overweight and obesity associating with higher odds of low birthweight and SGA. We also observed the odds of all six outcomes tended to increase as the degree of prepregnancy BMI increased above normal weight. Our findings emphasize the role of a healthy body weight for women of reproductive age, before conception, might play in mitigating the likelihood of infant adverse birth outcomes.

Our findings showed that both maternal prepregnancy underweight and overweight/obesity increased the odds of preterm birth, with the J-shaped relationships of prepregnancy BMI categories with preterm birth, which was independent of maternal age at delivery, race/ethnicity and infant birth year. However, previous studies on the association between maternal prepregnancy weight status and preterm birth has

TABLE 2 | Odds ratios and 95% CIs of infant birth outcomes according to maternal prepregnancy BMI categories.

Birth outcomes	Underweight	Normal weight	Overweight	Obesity grade 1	Obesity grade 2	Obesity grade 3
Preterm birth (<37 weeks)						
n (%)	29,679(9.5)	278,909(6.9)	177,198(7.2)	109,659(8.1)	57,470(8.8)	43,863(9.8)
Model 1	1.43(1.41–1.45)	1.00	1.06(1.05–1.07)	1.20(1.19–1.21)	1.31(1.30–1.32)	1.48(1.46–1.49)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 2	1.42(1.41–1.44)	1.00	1.03(1.02–1.03)	1.15(1.14–1.16)	1.25(1.24–1.26)	1.39(1.37–1.40)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 3	1.32(1.30–1.34)	1.00	1.04(1.04–1.05)	1.18(1.17–1.19)	1.31(1.29–1.32)	1.47(1.45–1.48)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Low birthweight (<2,500 g)						
n (%)	34,251(11.0)	247,138(6.1)	136,544(5.6)	79,574(5.9)	39,689(6.1)	28,970(6.5)
Model 1	1.84(1.82–1.86)	1.00	0.94(0.93–0.95)	1.01(1.00–1.02)	1.06(1.05–1.08)	1.16(1.14–1.17)
P-value	<0.0001		<0.0001	0.0127	<0.0001	<0.0001
Model 2	1.79(1.76–1.81)	1.00	0.90(0.89–0.91)	0.94(0.93–0.95)	0.98(0.97–0.99)	1.03(1.02–1.04)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 3	1.64(1.61–1.66)	1.00	0.79(0.78–0.80)	0.72(0.71–0.72)	0.65(0.64–0.66)	0.59(0.58–0.60)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Macrosomia (≥4,000 g)						
n (%)	8,718(2.8)	260,257(6.4)	218,481(8.9)	137,928(10.2)	75,351(11.6)	57,103(12.8)
Model 1	0.45(0.44–0.46)	1.00	1.43(1.42–1.43)	1.66(1.65–1.68)	1.92(1.90–1.93)	2.16(2.14–2.19)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 2	0.48(0.47–0.49)	1.00	1.49(1.48–1.49)	1.78(1.77–1.79)	2.06(2.04–2.07)	2.35(2.33–2.38)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 3	0.54(0.52–0.55)	1.00	1.53(1.52–1.54)	1.92(1.90–1.93)	2.33(2.31–2.35)	2.87(2.84–2.90)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
SGA (<P_{10})†						
n (%)	34,334(11.1)	254,306(6.3)	124,159(5.1)	65,399(4.9)	30,419(4.7)	19,844(4.5)
Model 1	1.81(1.79–1.83)	1.00	0.82(0.82–0.83)	0.80(0.79–0.81)	0.79(0.78–0.80)	0.77(0.76–0.78)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 2	1.70(1.68–1.72)	1.00	0.80(0.79–0.80)	0.75(0.75–0.76)	0.73(0.72–0.74)	0.69(0.68–0.70)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 3	1.56(1.54–1.58)	1.00	0.81(0.80–0.82)	0.77(0.76–0.77)	0.74(0.73–0.75)	0.69(0.68–0.70)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
LGA (>P_{90})†						
n (%)	7,177(2.3)	199,779(4.9)	183,242(7.5)	125,343(9.3)	72,877(11.2)	59,500(13.4)
Model 1	0.48(0.47–0.49)	1.00	1.55(1.54–1.56)	1.95(1.94–1.97)	2.40(2.38–2.42)	2.93(2.90–2.96)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 2	0.52(0.51–0.53)	1.00	1.60(1.59–1.61)	2.07(2.05–2.08)	2.56(2.54–2.58)	3.18(3.14–3.21)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 3	0.54(0.53–0.55)	1.00	1.58(1.57–1.59)	2.05(2.03–2.06)	2.54(2.52–2.56)	3.17(3.14–3.21)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Low Apgar score (<7 scores at 5 min)						
n (%)	5,134(1.7)	62,133(1.5)	42,052(1.7)	26,493(2.0)	14,892(2.3)	12,442(2.8)
Model 1	1.08(1.05–1.11)	1.00	1.12(1.11–1.14)	1.29(1.27–1.31)	1.51(1.48–1.53)	1.84(1.81–1.88)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001
Model 2	1.05(1.02–1.08)	1.00	1.12(1.10–1.13)	1.26(1.24–1.28)	1.44(1.42–1.47)	1.72(1.68–1.75)
P-value	0.0018		<0.0001	<0.0001	<0.0001	<0.0001
Model 3	0.88(0.86–0.91)	1.00	1.12(1.11–1.14)	1.21(1.19–1.23)	1.34(1.31–1.36)	1.55(1.51–1.58)
P-value	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001

BMI, body mass index; LGA, large for gestational age; SGA, small for gestational age.

† SGA and LGA were defined as birth weight below the 10th percentile (for SGA), and above the 90th percentile (for LGA) by gestational age and sex according to the U.S. new intrauterine growth curves.

Maternal pre-pregnancy normal weight was the reference group.

Model 1: Unadjusted model with only BMI as an explanatory variable.

Model 2: Adjusted for maternal age at delivery and race/ethnicity.

Model 3: Adjusted for maternal age at delivery, race/ethnicity, education levels, marital status, smoking status during pregnancy, live birth order, infant sex, gestational age (for low birthweight, macrosomia, or low Apgar score only), and the total number of prenatal care visits.

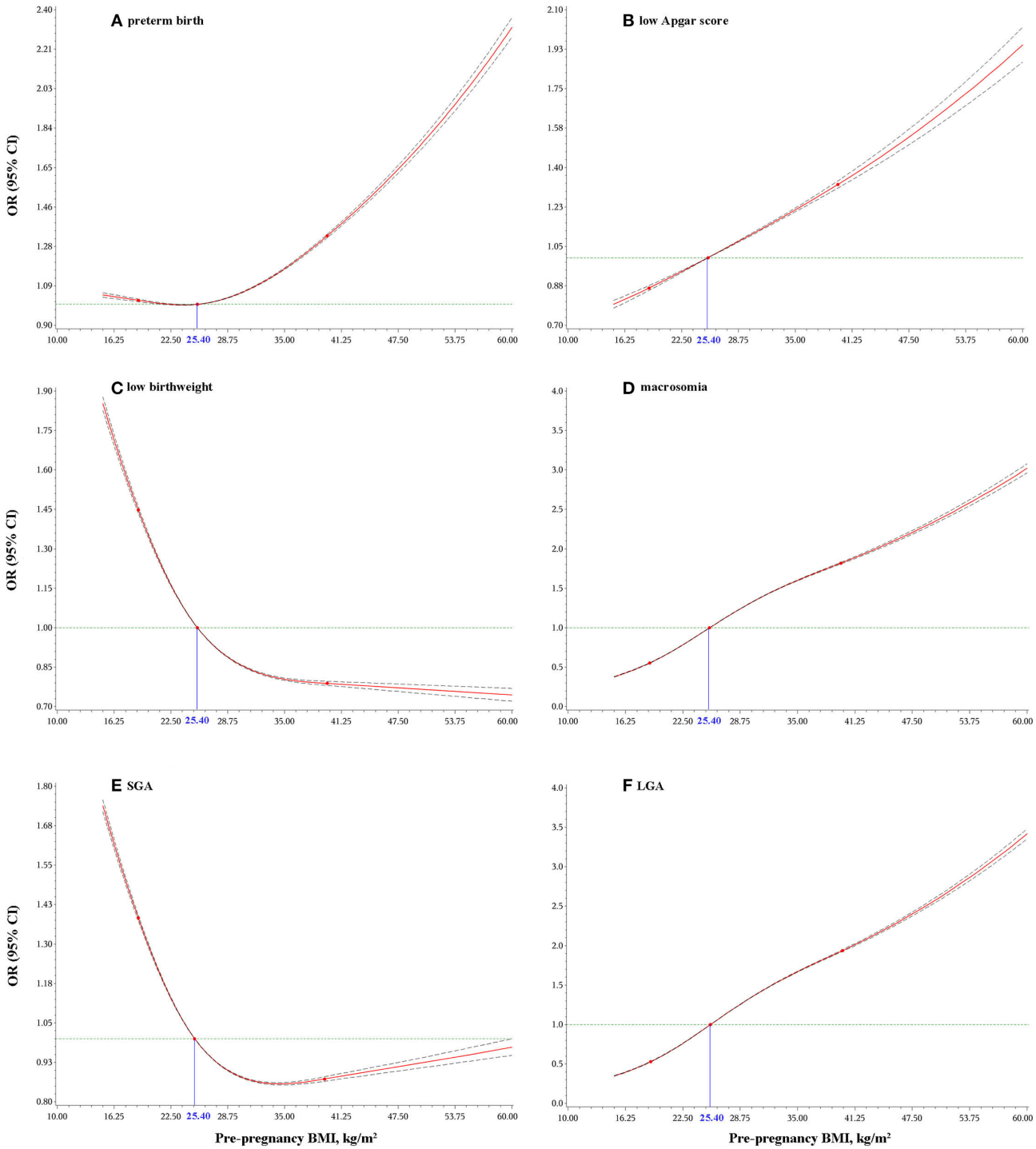

FIGURE 2 | Dose-response relationship of maternal pre-pregnancy BMI with: preterm birth **(A)**; low Apgar score **(B)**; low birthweight **(C)**; macrosomia **(D)**; small for gestational age (SGA) **(E)**; and large for gestational age (LGA) **(F)**. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated after adjusting for maternal age at delivery, race/ethnicity, education levels, marital status, smoking status during pregnancy, live-birth order, infant sex, gestational age (for low birthweight, macrosomia, or low Apgar score only), and total number of prenatal care visits.

been equivocal (6, 7, 16, 19–21). For example, a meta-analysis including 11 studies ($N = 452{,}991$) from 6 developing countries showed no significant association between prepregnancy overweight or obesity and preterm birth, and the author's observed high between-study heterogeneity (7). In addition, a meta-analysis including 21 studies ($N = 678{,}104$) from

China showed no significant association between prepregnancy underweight and preterm birth (21). The dissimilar conclusions may be because of the differences in selection of participants, sample size, race/ethnicity, maternal age at delivery, definition of weight categories, and other characteristics of the study populations.

We found that maternal prepregnancy overweight and obesity only increased the odds of macrosomia and LGA rather than low birthweight and SGA, which was consistent with the conclusions of some meta-analyses (8, 22). However, some studies reported that women with prepregnancy obesity had an increased likelihood of both macrosomia and low birthweight (8, 23) or both LGA and SGA (9). Inconsistencies in observed results might be because of the participant selection, statistical power, characteristics of the study populations, and adjustment for different covariates. For example, the association between prepregnancy obesity and low birthweight was substantially reduced and no longer significant after adjustment for gestational age and other maternal factors in a prospective cohort study (24). Based on the largest sample size examined to date, and the use of national birth certificate data, our dose–response analysis suggested that prepregnancy BMI at the lower extreme increased the odds of low birthweight and SGA, and at the upper extreme increased the odds of macrosomia and LGA. Moreover, we also found that women with a prepregnancy normal BMI range of 18.5–25.0 kg/m^2 in this observed population had higher odds of low birthweight and SGA, indicating that a prepregnancy BMI within this range is not devoid of risk, but this needs further investigation and confirmation—perhaps considering additional factors (e.g., contribution of father or diet quality of pregnant mothers) that might confound or act as an effect modifier of the association.

Our finding showed that there were significant associations between maternal prepregnancy overweight and obesity and low Apgar score. This finding is in accord with a meta-analysis that considered data from 11 studies (N = 2,586,265 participants) where maternal overweight and obesity were associated with higher odds of low Apgar scores (10). However, data from some individual cohorts have shown no significant or a weak association between prepregnancy obesity and low Apgar score (11, 25, 26). In addition to the lower statistical power of individual studies compared with meta-analysis, varying measurement of, and adjustment for, potential confounders may also explain these inconsistent findings.

Maternal prepregnancy BMI categories at both upper and lower extremes may affect infant adverse birth outcomes in several ways. First, prepregnancy overweight and obesity usually cause metabolic abnormalities during pregnancy [such as gestational hypertension and diabetes (6, 7)] which may lead to placental abnormalities (27–31), and ultimately affect adverse birth outcomes. Second, excessive or poor maternal periconceptional weight status may increase the risk of abnormal growth and development of the offspring through epigenetic imprinting or methylation (32–34). Third, prepregnancy overweight and obesity may cause the imbalance of maternal intestinal microbiota (35, 36) that may impose an adverse effect on birth outcomes (37).

Our study has several strengths. First, we used the largest sample size with a total of more than 9 million mother–infant pairs from national birth certificate data collected in 50 states and the District of Columbia of the U.S. between 2016 and 2018. Second, we examined a wide range of adverse birth outcomes including preterm birth, low birthweight, macrosomia, SGA, LGA, and low Apgar score. Third, we performed dose–response relationship analysis to assess the stability of our findings by excluding women with cesarean section, and those with eclampsia, gestational hypertension, or diabetes. Fourth, we had data on many potential confounders that allowed us to adjust on the basis of them. However, our study also has limitations. First, maternal prepregnancy BMI was calculated based on self-reported weight and height before pregnancy, but the accurate representation of objective BMI (calculated using measured weight and height) among U.S. women of reproductive age has been shown (38). Second, we did not analyze the association of gestational weight gain with adverse infant birth outcomes in this study since previous studies showed that gestational weight gain presented a weaker association than prepregnancy BMI (39).

CONCLUSION

Based on a very large US cohort, we examined the associations between maternal prepregnancy BMI and a number of birth adverse outcomes and performed the dose–response analysis. We found that maternal prepregnancy overweight and obesity associated with the higher odds of preterm birth, macrosomia, LGA, and low Apgar score; maternal prepregnancy underweight is associates with higher odds of preterm birth, low birthweight, and SGA. In consideration of the increasing prevalence of obesity among women of reproductive age worldwide, our findings highlight early health education and implementation of healthy weight management among women planning a pregnancy could substantially reduce the health burden posed by adverse infant birth outcomes.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BX conceived and designed the study and supervised the study. HW and LY conducted all the analyses.

XZ, CM, and BX wrote the manuscript. All the authors carried out the study and interpreted the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.789833/full#supplementary-material

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