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Developmental origin of diseases: A special focus on the parental contribution towards offspring's adult health

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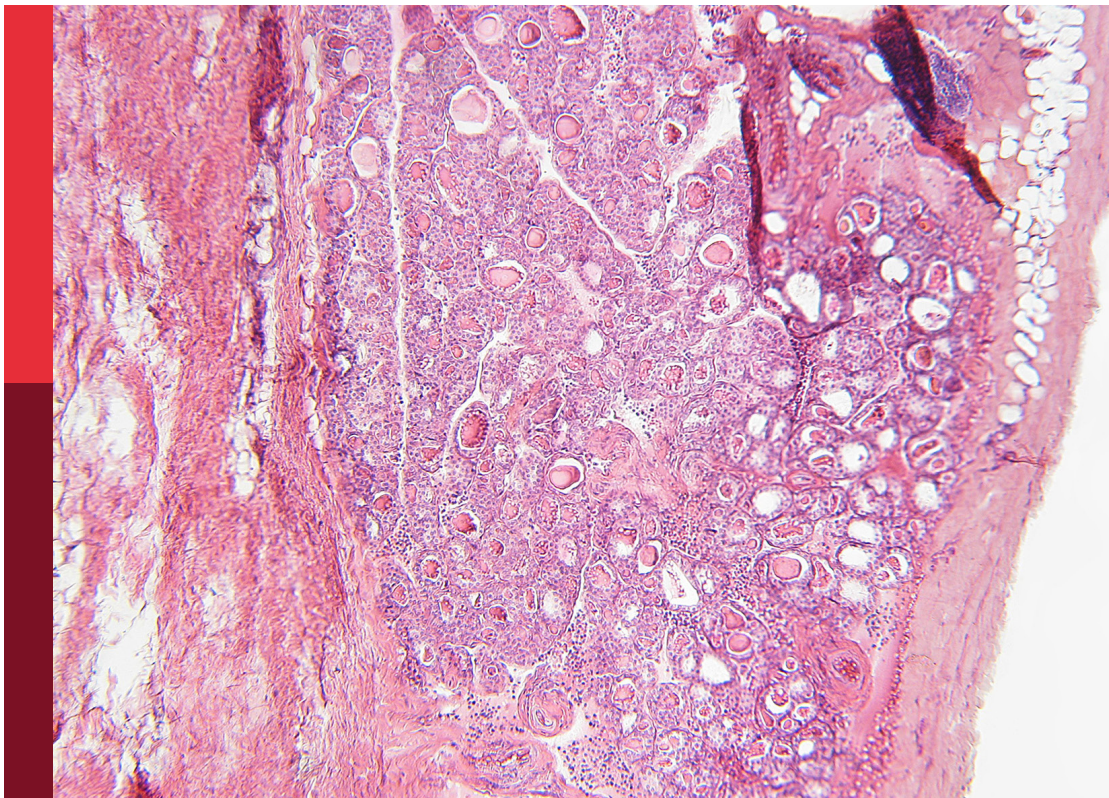

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Developmental origin of diseases: A special focus on the parental contribution towards offspring's adult health

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Editorial: Developmental origin of diseases: a special focus on the parental contribution towards offspring's adult health

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Editorial on the Research Topic
Developmental origin of diseases: a special focus on the parental contribution towards offspring's adult health

The developmental origins of health and disease (DOHaD) hypothesis proposes that early life experiences can impact an individual's health and disease risk throughout their lifespan. While much research in this field has focused on the role of maternal factors, recent studies have increasingly turned attention to how paternal factors can also influence offspring health outcomes. The special issue on "Developmental Origin of Diseases: A Special Focus on the Parental Contribution Towards Offspring's Adult Health" in Frontiers in Endocrinology features 10 articles that examine various aspects of the complex relationship between parental factors and offspring health outcomes. The articles cover a broad range of topics, from the effects of maternal obesity and paternal diet on offspring health to the impact of parental smoking and mental health on offspring metabolic and mental health outcomes.

Several articles explore the link between maternal factors and offspring health outcomes. For instance, Sugino et al. revealed the association between maternal diet, bacterial diversity, and infant pathogenicity. In particular, this manuscript underlines that the presence of complex carbohydrates in the diet of women with gestational diabetes mellitus (GDM) leads to bacterial diversity in the infants, which could reduce the pathogenicity in the starting months of the infant's life.

The transmission of epigenetic changes to the offspring is associated with various health issues. In this regard, Wang et al. studied the placental epigenome of GDM. The study found 256 differentially methylated positions between the control and GDM groups. Further, the study revealed the importance of 11 genes (*CYP2D7P1, PCDHB15, ERG, SIRPB1, DKK2, RAPGEF5, CACNA2D4, PCSK9, TSNARE1, CADM2, KCNAB2*) related to fetal growth.

Intrauterine growth restriction leads to health adversities later in life. In this line, Doan et al. provide an in-depth review of intrauterine growth restriction, epigenetic alteration, and its impact on fetal development. This review exhaustively covers the altered epigenetic mechanisms in different tissues such as fetal blood, umbilical cord, and placenta for small gestational age babies. Moreover, this review also discusses that intrauterine growth restriction phenotypes can be transferred in subsequent generations and possibly associated epigenetic mechanisms.

Gestational diabetes is a common condition affecting a significant proportion of women globally. Vasilakos et al. examined the levels of umbilical cord blood leptin and IL-6 in the presence of maternal diabetes or chorioamnionitis. The findings of the study suggest that maternal diabetes during pregnancy is associated with increased leptin and IL-6 levels in the cord blood, which could potentially lead to metabolic dysfunction in offspring. These results highlight the importance of managing diabetes during pregnancy to reduce the risk of future metabolic disorders in offspring.

Another study by Yang et al. confirmed that a maternal high-fat diet during the pre-weaning period impacts the offspring's metabolic programming. The study also found impairments of brown adipose tissue thermogenesis due to beta-adrenergic signaling in the mice that consumed a high-fat diet. Bukowski et al.'s study is centered on demonstrating the role of transient receptor potential canonical channel 1 (TRPC1) on lipid homeostasis. TRPC1 is an integral protein that regulates Ca2+ ion flux across the membrane, and the study revealed that the removal of TRPC1 showed increased adiposity in the high-fat diet consumed mouse.

Birth weight is considered a fetal growth marker *in utero*, and a retrospective study by Mao et al. on 69,000 hospital deliveries found that advanced parental age is linked to small and large gestation ages in preterm and term infants, respectively. Proper liver functioning is crucial for detoxification, nutrient absorption, and metabolism. Sarli et al. investigated the impact of restricted maternal food intake on the liver proteome of the offspring using Wistar rats. The Wistar rats were divided into three groups: 1) control (normal laboratory diet), 2) offspring born with 50% restricted maternal diet (FGR group, low birth weight), and 3) offspring appropriately grown/born with 50% restricted maternal diet (non-FGR group). The study found differential expression of 451 and 751 liver proteins between control vs. FGR and non-FGR, respectively, which are involved in cholesterol biosynthesis, thyroid hormone metabolism, fatty acid beta-oxidation, and apelin liver metabolic pathways.

Nuclear receptors have a vital biological function. Zhu et al. found a mutation in NR0B1 (nuclear receptor subfamily 0 group B member 1 gene) associated with congenital adrenal hypoplasia and pubertal development failure in an adult male, while a case report by Lei et al. found a deletion of the 6p25.3p25.2 region containing 28 protein-coding genes such as *IRF4, FOXF2, TUBB2B, and FOXC1* is associated with congenital phenotypes in a neonate through chromosomal analysis.

Due to industrial and agricultural development, thousands of chemicals contaminate the different environmental compartments, such as air, food, and water, directly or indirectly impacting organism health, including humans. Hsu and Tain comprehensively reviewed the impact of toxicants on chronic kidney disease and hypertension, providing epidemiological and experimental evidence that underlines the potential renal toxicants, prenatal chemical exposure, their major sources, associated renal toxicity, and therapeutic interventions.

The articles in this special issue cover a broad range of topics, from the effects of maternal obesity and paternal diet on offspring health to the impact of parental smoking and mental health on offspring metabolic and mental health outcomes. By shedding light on the role of both maternal and paternal factors in shaping offspring health, these articles offer valuable insights into the developmental origins of health and diseases and its potential implications for clinical practice and public health policy.

Author contributions

AS: Conceptualization; Writing - original draft. MM: Writing - review & editing. KS: Conceptualization; Writing - review & editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Liver Proteome Profile of Growth Restricted and Appropriately Grown Newborn Wistar Rats Associated With Maternal Undernutrition

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Background: Fetal growth restriction (FGR) has been associated with adverse perinatal outcomes and epigenetic modifications that impact gene expression leading to permanent changes of fetal metabolic pathways and thereby influence development of disease in childhood and adult life. In this study, we investigated the result of maternal food restriction on liver protein expression in Wistar male newborn pups.

Materials & Methods: Ten (n = 10) timed pregnant Wistar rats on their 14th day of gestation were randomly assigned to either control (n = 4) or food restricted group (n = 6). The control group had *ad libitum* access to food. In the food restricted group, maternal diet was limited in a moderate fashion (50%) from day 15 of pregnancy until delivery. All rats delivered spontaneously on day 21 and newborn pups were immediately weighed. Pups born to normally nourished mothers were considered as controls, while pups born to food restricted mothers were subdivided into two groups, based on their birth weight: growth restricted (FGR) and appropriately grown (non-FGR). Rats were euthanized immediately after birth and liver tissues of 11 randomly selected male offspring (FGR n = 4, non-FGR n = 4, control n = 3) were collected and analyzed using quantitative proteomics.

Results: In total 6,665 proteins were profiled. Of these, 451 and 751 were differentially expressed in FGR and non-FGR vs. control, respectively, whereas 229 proteins were commonly expressed. Bioinformatics analysis of the differentially expressed proteins (DEPs) in FGR vs. control revealed induction of the super-pathway of cholesterol biosynthesis and inhibition of thyroid hormone metabolism, fatty acid beta oxidation and apelin liver signaling pathway. Analysis of DEPs in non-FGR vs. control groups showed inhibition of thyroid hormone metabolism, fatty acid beta oxidation, and apelin liver signaling pathway.

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Conclusion: This study demonstrates the impact of prenatal food restriction on the proteomic liver profile of FGR and non-FGR offspring underlying the importance of both prenatal adversities and birth weight on liver-dependent postnatal disease.

Keywords: FGR, fetal programming, food restriction, metabolic disorders, liver proteomics

INTRODUCTION

Fetal Growth Restriction (FGR) refers to a fetus that has failed to achieve its biological growth potential due to pathological conditions such as maternal/fetal disease and placental dysfunction. Fetal growth impairment is associated with perinatal morbidity and mortality, a 5- to 10-fold risk of *in utero* demise (1) and adverse neonatal outcomes (2). Furthermore, according to Barker's hypothesis, an unfavorable intrauterine environment may have negative long-term effects in adult life (3). According to the thrifty phenotype hypothesis (4), FGR impairs the growth of organs such as the liver in order to maintain homeostasis of other crucial for survival organs and systems. These metabolic adaptations, enable fetuses to survive in a malnourished intrauterine environment. However, the cost of these adaptations is permanent physiological and epigenetic phenotypical alterations that are responsible for development of disease later in life such as obesity, diabetes, and cardiovascular disease (5).

Nutrition is one of the environmental variables with the widest range of effects on both physical growth and metabolism (6, 7). An expanding body of epidemiological evidence suggests that the nutritional environment experienced in fetal life increases the risk of chronic non-communicable diseases associated with human ageing. Maternal undernutrition constitutes a serious public health problem exhibiting large regional and within-country variations across the globe. Proper nutrition from preconception to delivery is critical for avoiding poor pregnancy and long-term outcomes for both the mother and child (8). A human model of fetal programming regarding the effects of maternal malnutrition on development of postnatal disease has been illustrated by the Dutch cohort from the Hunger Winter of 1944. It involved pregnant mothers exposed to famine and its long-term consequences for adult health. Poor maternal nutrition during gestation was associated with a higher prevalence of atherogenic lipid profile, obesity, insulin resistance, and cardiovascular disorders (9). To date, many experimental approaches have been designed to study the impact of FGR intervening either in maternal nutrition, placental blood flow or fetal wellness. Restricting maternal food intake is advantageous since it leads to an altered intrauterine nutritional milieu and growth impairment avoiding surgical intervention. Moreover, this type of animal model is closer to pregnancy malnutrition effects observed in humans (10, 11). Although a large number of animal models of FGR have investigated the impact of intrauterine environment on fetal epigenetic programming, there is little knowledge about the effects of maternal undernutrition on liver growth and physiology of appropriately grown (non-FGR) offspring of undernourished pregnancies.

Liver plays a major role in nutrients' absorption and metabolism. During pregnancy, fetal growth restriction not only affects adversely liver's growth but also its physiological function (12). Metabolic disorders namely, reduced oxidative phosphorylation, impaired mitochondrial function, antioxidant capacity, and altered nutrient metabolism are commonly found in FGR livers (13–15). It has been demonstrated that liver of FGR offspring seems to have an abnormally increased rate of gluconeogenesis contributing to insulin resistance and hyperglycemia (16, 17). Nevertheless, the exact mechanisms which are responsible for alterations in development, growth, and liver function leading to hepatic diseases are not adequately described. Previous animal studies have shown that maternal undernutrition and consequent FGR alters effectively the liver proteome through altered activities of many key enzymes (18). Proteomic studies in piglets revealed that many liver proteins involved in oxidative stress, intermediate metabolism, cell structure, and growth were differentially expressed in FGR offspring. Furthermore, nutritional models of fetal programming indicated that caloric restriction and low birth weight are strongly related with epigenomic changes in liver leading to insulin resistance and NAFLD (19). Our aim was to investigate the impact of maternal food deprivation on liver proteomic profile in three groups of newborn male Wistar rats: a) offspring of mothers that received standard laboratory diet (control group), b) offspring of food restricted mothers with low birth weight (FGR group), and c) appropriately grown offspring of food restricted mothers (non-FGR group).

Furthermore, the aim of this study was to examine whether prenatal food restriction during late gestation affects offspring liver proteome irrespective of birth weight and propose possible underlying pathophysiological mechanisms of liver fetal programming.

MATERIALS AND METHODS

Animal Model

Ten (n = 10) timed pregnant Wistar rats, on their 12th day of gestation (Janvier Labs – Rodent research models & associated services, France), were hosted individually in 36 × 20 × 14 cm breeding boxes at the Laboratory of Experimental Surgery of the Second Department of General Surgery at Aretaieion Hospital, National and Kapodistrian University of Athens, Athens, Greece. Animals were housed under standard laboratory conditions (temperature between 22°C and 23°C, humidity 55% to 65% and 12-h light/dark cycles). All animals were fed with standard formula diet containing 18.5% protein (Mucedola S.r.l., Settimo Milanese, Italy) with ad libitum access to food and water as well,

until day 14. Following randomization, pregnant dams were assigned to starved group (n = 6, diet restricted by 50%) and control group (n = 4 ad libitum access to food). Both groups had free access to fresh water. Control group's food intake was measured on a daily basis. During the experimental period (from day 15th onward), rats of the starved group, were given half the amount of food that was on average consumed by the control group, based on measurements taken place the day before. Food restriction of the starved group lasted from 15th gestational day to delivery. All rats delivered spontaneously on the 21st gestational day and neonates were immediately weighted (**Figure 1**). Starved group's offspring were categorized according to their birth weight as FGR (birth weight < mean birth weight of control group's offspring − 2 × standard deviation) and non FGR (birth weight > mean birth weight of control group's offspring − 2 × standard deviation) as previously described (20–24). Immediately after delivery, offspring were separated from their mothers and weighted. Neonates were anesthetized using inhaled sevoflurane, and euthanized. Liver tissues were rapidly removed. The time interval between rat's sacrifice and specimens' storage at −80°C did not exceed 15 min. All liver tissues were cleaned from blood with PBS (phosphate buffered saline). Specimens were stored at −80°C and sent packed in dry ice to the Centre for Proteomic Research, Institute for Life Sciences, University Southampton for proteomic analysis. Growth characteristics of mothers, gestation duration, litter size, birth weight of the pups, and organ weight were compared using the independent-samples t-test (IBM SPSS Statistics 22.0). Statistical significance was considered at $p < 0.05$. The animal model and study design have been previously presented and data on heart and brain proteomic analysis have been published (22, 24).

This study received ethics approval by the Ethics Committee of Aretaieion University Hospital, Medical School of the National and Kapodistrian University of Athens with registration number B 207/13-10-2016. Research license and approval for experimental animal (RjHan : WI – Wistar rats)

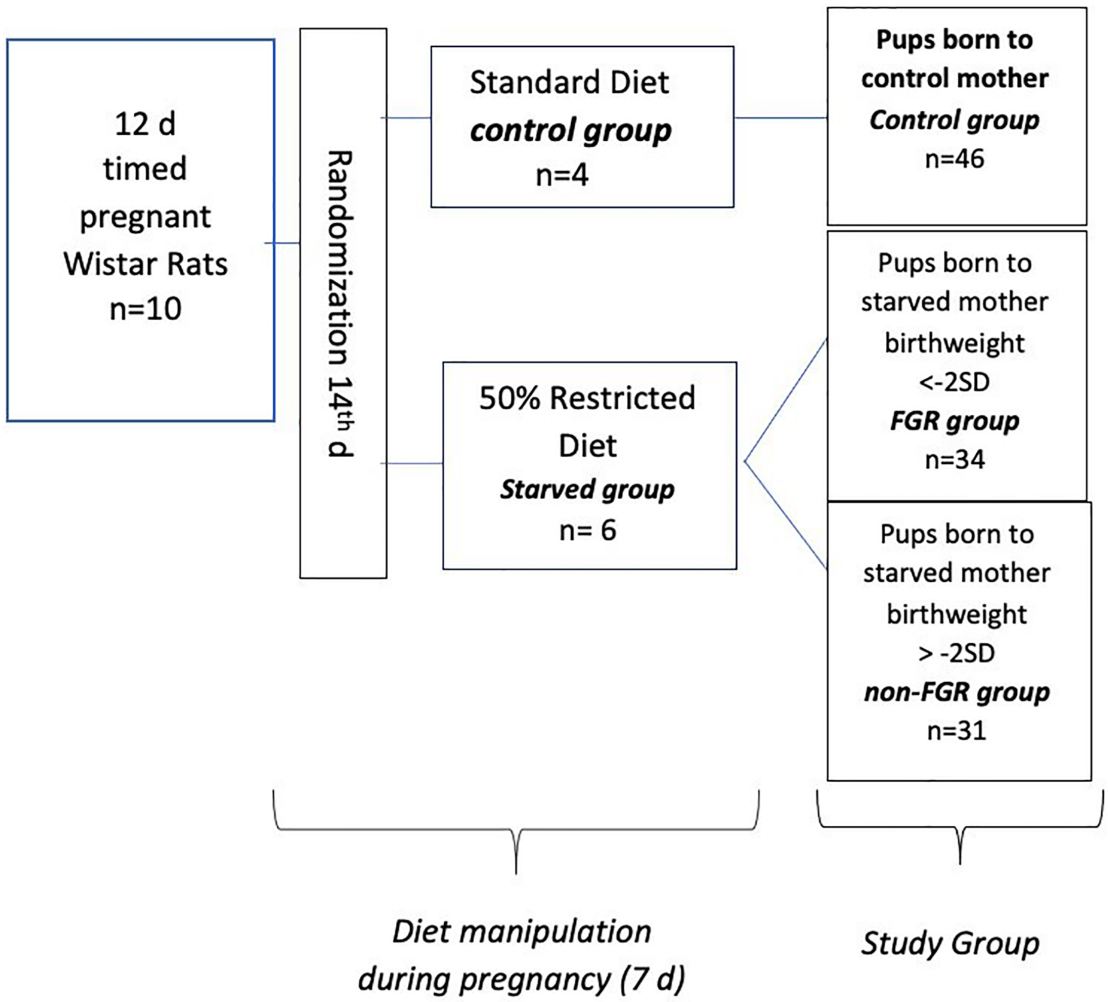

FIGURE 1 | Experimental design of the study.

utilization was granted by the Division of Agriculture and Veterinary Policy, District of Attica, Greece (Decision 5035/21-09-2017 and its modification 1211/19-03-2018). Animal handling was performed in accordance with the local applied laws (1197/1981 and 2015/1992) for the protection of animals and the Directive 2010/63/EU of the European Parliament and Council regarding the protection of animals used for research purposes. Based on the Directive 2010/63/EU of the European Parliament and Council, stating that animals should experience the minimum pain, suffering, and distress, we used inhalant sevoflurane in overdose as a euthanasia method.

Quantitative Proteomics

Each liver tissue was dissolved in 200 µl of 0.5 M triethylammonium bicarbonate, 0.05% sodium dodecyl sulphate and homogenized using the FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, CA, USA). Lysates were subjected to pulsed probe sonication (Misonix, Farmingdale, NY, USA) and centrifuged (16,000 g, 10 min, 4°C). The supernatant had been measured for its protein content using the Direct Detect™ system (Merck Millipore, Darmstadt, Germany). From each lysate, 100 µg of protein subjected to reduction, alkylation, trypsin proteolysis, and 11-plex TMT labelling according to manufacturer's instructions. The resulting TMT peptides were initially fractionated with alkaline C_4 reversed phase (RP) liquid chromatography. Each peptide fraction further separated with on-line nano-capillary C_{18} reverse phase liquid chromatography under acidic conditions, subjected to nanospary ionization, and measured with ultra-high resolution mass spectrometry using the hybrid ion-trap/FT-Orbitrap Elite platform. The unprocessed raw data files were submitted to Proteome Discoverer 1.4 for target decoy searching with SequestHT against the TREMBL Uniprot database for Rattus norvegicus (release date: January 2018). Reporter ion ratios derived from unique peptides only were used for the relative quantitation of each respective protein. Quantification ratios were median-normalized and $\log_2$ transformed. The threshold of percent co-isolation excluding peptides from quantification was set at 50. A one-sample T-Test was performed to identify proteins that were differentially expressed in the tissue from FGR and non-FGR compared to control rats. The two-stage step-up method of Benjamini, Krieger, and Yekutieli was used for multiple hypothesis correction. A $q\text{-}value \leq 0.05$ was considered significant. Proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository.

RESULTS

Experimental Model

Our experimental study consists of 111 newborn pups, which are divided into two sub-groups; the starved and the control group (food restricted group vs. control group; n = 65, 58.6% *vs.* n = 46, 41.4%), 57 offspring (51.4%) were male (22 in the control group and 35 in the food restricted group) and 54 (48.6%) were female (24 and 30), respectively. There was no statistical difference in post-delivery maternal bodyweight in both diet groups (control: 265 ± 25 gr, starved 270 ± 20 gr p = 0.769). Control group mothers (ad libitum food access) gave birth to control pups with mean body weight of 6.419 gr (SD: 0.436). The mean birthweight of the food restricted group was 5.423 gr, significantly different compared to controls (5.423 ± 0.610 gr *vs.* 6.419 ± 0.436 gr; p<0.001). Male neonates were heavier compared to females in the control group (6.659 g *vs.* 6.2 g, p < 0.001) but there was no statistically significant difference between them in the starved group (p = 0.666). Newborn pups delivered by starved mothers, were further divided to Fetal Growth Restricted (FGR) group when birthweight was < - 2SDs of the mean BW of the control offspring and non-FGR group when birthweight was > - 2SDs of the mean BW of the control. The cut-off between FGR and non-FGR neonates was set at 5.547 gr according to the aforementioned definition. Furthermore, there was statistically significant birthweight difference between FGR (4.796 gr ± 0.479 gr) and non-FGR (5.914 gr ± 0,479 gr) groups (p<0,001). Food restricted group reveals a remarkable sex differentiation impact on birth weight.

Even though male pups were heavier at birth compared to females in both the control group (control males vs. control females; 6.659 ± 0.324 g *vs.* 6.200 ± 0.413 g; p<0.001) and the non-FGR group (non-FGR males *vs.* non-FGR females; 5.930 ± 0.298 g. *vs.* 5.880 ± 0.131 g; p = 0.519), FGR male newborns weight 8.5% less than the female ones (FGR males *vs.* FGR females; 4.739 ± 0.629 g *vs.* 5.142 ± 0.240 g; p<0.05). Following this observation and in order to avoid bias due to sex differentiation we decided to include only male offspring for quantitative proteomic analysis.

Although liver weight of the non-FGR group was statistically significant higher compared to FGR pups (0.211 ± 0.047 *vs.* 0.280 ± 0.073, p < 0.0014), there was no difference in the liver weight to body weight ratio between groups (0.04274 ± 0.00743 *vs.* 0.04721 ± 0.01220, p = 0.10337) (**Tables 1**, **2**).

Proteomic Analysis

Proteomic analysis of male offspring livers ended up in the profiling of 6,665 proteins (peptide level q<0.05) (**Supplementary Table 1**). Among the quantified proteins, 451 proteins were differentially expressed in FGR *vs.* control (**Supplementary Table 2**) and 782 in non-FGR *vs.* control group (**Supplementary Table 3**). Of these, 76 were commonly up-regulated and 153 commonly down-regulated in both FGR and non-FGR compared to control (**Supplementary Table 4**) (**Figure 8**). Principal component analysis (PCA) of all quantified proteins showed a distinct proteomic liver profile of FGR compared to non-FGR rats (**Figures 2**, **3**). Bioinformatics analysis of differentially expressed proteins (DEPs) in FGR compared to control groups using Ingenuity Pathway Analysis (IPA) showed: a. induction of the super pathway of cholesterol biosynthesis (z = 2.2; p = 1.5e-4) (**Figure 4**), and b. inhibition of thyroid hormone metabolism (**Figure 5**) (z = −2.0; p = 4.6e-3), fatty acid beta oxidation (z = −2.0; p = 2.7e-3) (**Figure 6**), and apelin liver signaling pathway (**Figure 7**) (z = −2.2; p = 8.5e-5). Enrichment analysis of the DEPs in non-FGR vs. control groups

TABLE 1 | Mean values for all experimental outcomes and comparisons between study groups.

	Group	Mean	p-value
Length of gestation (days)	***Starved***	21.22 ± 0.47	0.081
	Control	20.73 ± 0.06	
Litter size (pups) (n = 111)	***Starved***	10.83 ± 1.72	0.530
	Control	11.50 ± 1.29	
Post-delivery maternal weight(g) (n = 10)	***Starved***	268.83 ± 26.75	0.304
	Control	264.50 ± 10.47	
Birth weight (g)	***Starved***	5.423 ± 0.610	<0.001
	Control	6.419 ± 0.436	
Liver weight (g)	***Starved***	0.245 ± 0.070	0.117
	Control	0.266 ± 0.057	
	Fgr	0.211 ± 0.047	<0.001
	non-Fgr	0.280 ± 0.073	
Liver to body weight (g)	***Starved***	0.04498 ± 0.01026	0.112
	Control	0.41753 ± 0.00946	
	FGR	0.04274 ± 0.00743	0.103
	non-FGR	0.04721 ± 0.00743	
Brain weight (g)	***Starved***	0.150 ± 0.045	<0.001
	Control	0.180 ± 0.044	
	FGR	0.151 ± 0.058	0.783
	non-FGR	0.148 ± 0.043	
Brain to body weight (g)	***Starved***	0.02826 ± 0.00968	0.905
	Control	0.02806 ± 0.00598	
	FGR	0.03157 ± 0.01093	0.009
	non-FGR	0.02496 ± 0.00698	

TABLE 2 | Birth and liver tissue mean weights of the newborn pups in control, food restricted group and both subcategories of starved group.

	Control Group	Starved Group	FGR	Non-FGR
Birth weight				
Male (n = 57)	6.659 ± 0.324	5.454 ± 0.744*	4.739 ± 0.629*	5.930 ± 0.298*
Female (n = 54)	6.200 ± 0.413	5.388 ± 0.410*	5.388 ± 0.410*	5.880 ± 0.131**
Liver weight				
Male	0.296 ± 0.028	0.240 ± 0.077*	0.173 ± 0.038	0.291 ± 0.056
Female	0.271 ± 0.059	0.250 ± 0.061*	0.222 ± 0.030	0.3 ± 0.069
Liver to body weight				
Male	0.05911 ± 0.08464	0.04438 ± 0.00453*	0.04311 ± 0.00765	0.04929 ± 0.00905
Female	0.04407 ± 0.01095	0.04594 ± 0.00941**	0.04303 ± 0.00519	0.051 ± 0.01212

**p-value < 0.001, **p-value < 0,01 (compared to control group).*

using IPA showed: a. induction of immune cell adhesion (z = 2.9; p = 1.1e-7) and b. inhibition of thyroid hormone metabolism (z = −2.0; p = 2.5e-2), fatty acid beta oxidation (z= -2.0; p = 1.6e-2) and apelin liver signaling pathway (z = −2.0; p = 6.7e-3) (**Figure 8**).

DISCUSSION

Numerous studies have shown the impact of adverse early-life environment on disease during infancy, childhood, and adult life (25, 26). Fetal growth restriction is associated with significant perinatal and subsequent long-term morbidity and mortality (27). FGR neonates and infants demonstrate a variety of complications involving multiple organs and systems such as pulmonary, gastrointestinal, immune, and central nervous system. Regarding the endocrine system, FGR is associated with altered glucose metabolism, transiently low thyroxin levels and cortisol deficiency. Furthermore, FGR programs both childhood and adult disease, associated with increased risk of obesity, insulin resistance, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (13, 28, 29).

Using a well-defined FGR rat model, this study shows that maternal food restriction plays a crucial role, impairing liver intrauterine growth and altering its proteomic expression. In our study liver weight was reduced in proportion to body weight in FGR compared to non-FGR pups. On the contrary, brain weight did not differ significantly between the abovementioned two groups (**Table 1**) indicating a late-onset FGR model resembling to the commonest FGR phenotype in human population (30). This study aimed to a better understanding of the proteomic mechanisms of liver developmental dysfunction induced by prenatal food restriction investigating possible differentiations in liver proteomic expression in both growth restricted (FGR) and appropriately grown (non-FGR) offspring born to starved mothers. To our knowledge, this study is the first one to report the proteomic profiling of liver in both FGR and non-FGR Wistar rat offspring exposed to prenatal food restriction. Our

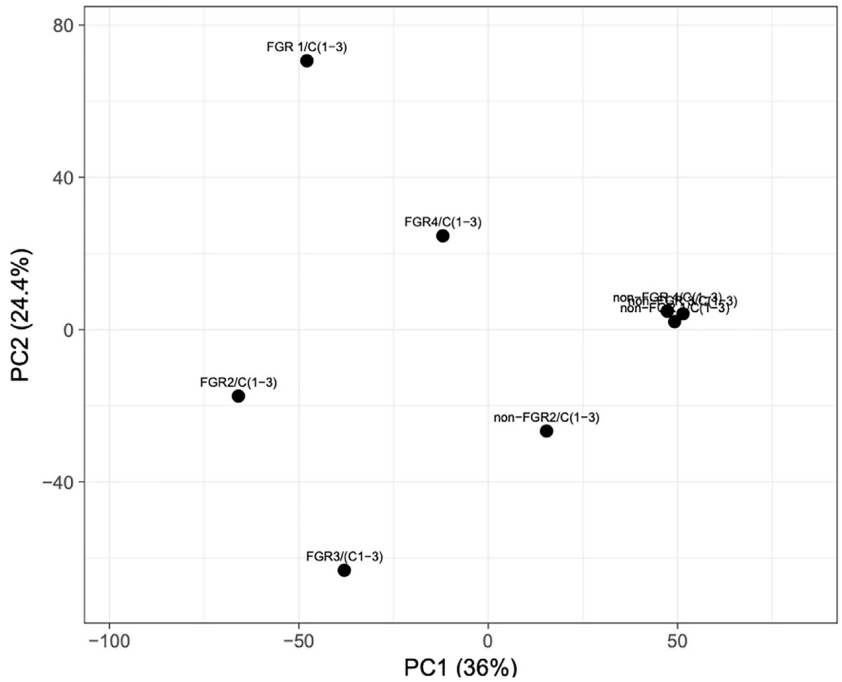

FIGURE 2 | Principal component analysis (PCA) of all quantified proteins revealed that liver of fetal growth restricted pups had a heterogeneous proteomic profile compared to non-FGR ones.

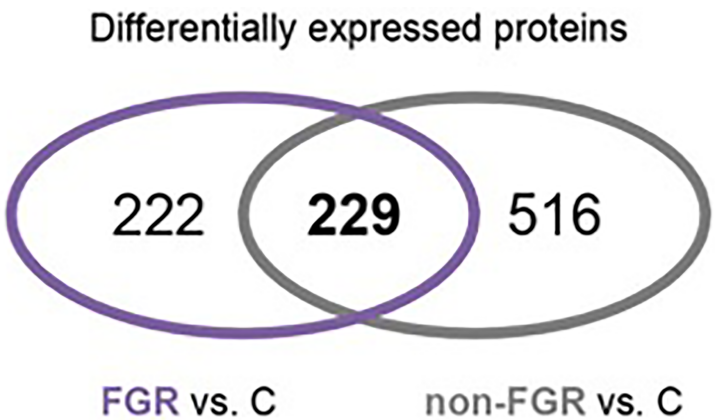

FIGURE 3 | Venn diagram of common Differentially expressed proteins in FGR *vs.* Control and non-FGR *vs.* Control group.

study demonstrated that maternal undernutrition produced a distinct proteomic profile in FGR and non-FGR pups. These changes are indicative of an induction of cholesterol biosynthesis and inhibition of thyroid hormone metabolism, fatty acid beta oxidation, and apelin liver signaling

Bioinformatic analysis of DEPs in the FGR group vs. control showed induction of cholesterol biosynthesis. Regarding cholesterol biosynthesis, metabolomic studies have shown that FGR fetuses have higher concentrations of cholesterol such as VLDL and LDL, lipoproteins, and triglycerides (31). Lipids are vital molecules for life, providing energy for metabolic processes. Furthermore, cholesterol is a key element for brain neurodevelopment and a precursor of many hormones like sex steroids (32, 33). Fetal liver is the main source of circulating lipoproteins, as in adults. Alterations of VLDL concentrations, which are mostly synthesized in fetal liver, imply an altered

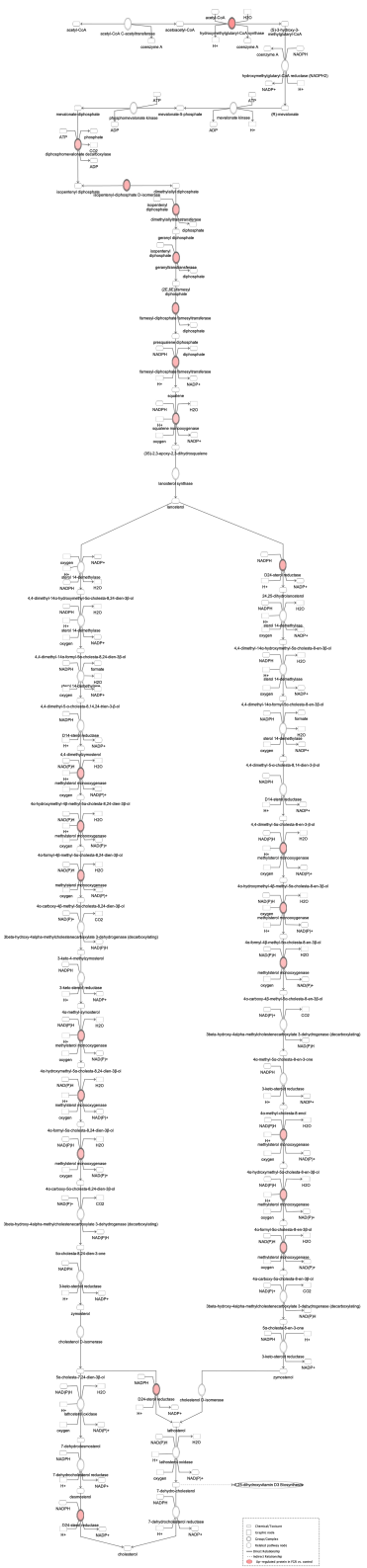

FIGURE 4 | Ingenuity Pathway Analysis of DEPs between FGR *vs.* Control group. *Induction of the super pathway of cholesterol biosynthesis*. **(z = 2.2, p = 1.5e-4)**.

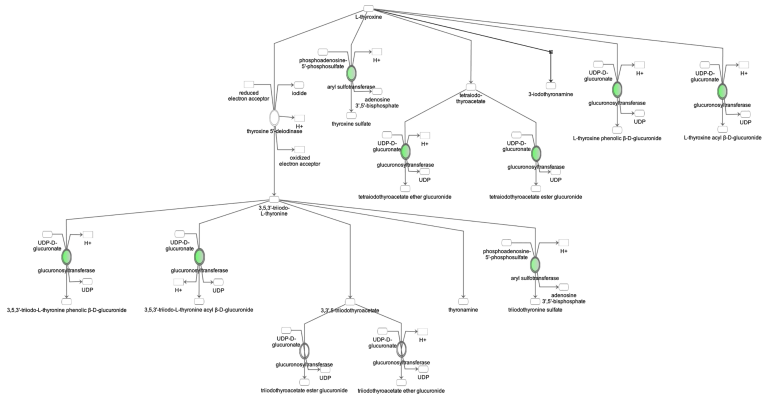

FIGURE 5 | Ingenuity Pathway Analysis of DEPs between FGR vs. Control group. *Inhibition of thyroid hormone metabolism.* **(z = -2.0, p = 4.6e-3)**.

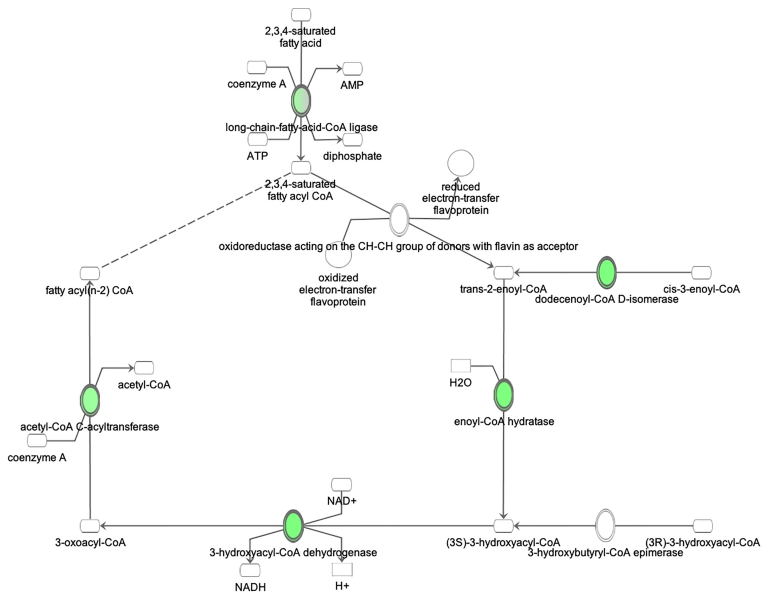

FIGURE 6 | Ingenuity Pathway Analysis of DEPs between FGR *vs.* Control group. *Inhibition of fatty acid beta oxidation.* **(z = –2.0, p = 2.7e-3)**.

hepatic synthesis of lipoproteins caused by FGR. Remarkably, the lipid profile of FGR fetuses resembles to adults presenting with atherosclerosis and dyslipidemia (34, 35).

The apelin signaling pathway, thyroid metabolism, and fatty acid beta oxidation were inhibited in both FGR and non-FGR neonate rats, indicating these might be a result of maternal undernutrition regardless the fetus' growth. Apelin is a regulatory peptide and in conjunction with its receptor, are both expressed in a wide range of tissues such as central nervous system, heart, and liver. Apelin is also produced by adipocytes and latest studies proposed its crucial role in energy metabolism and enhancement of insulin sensitivity (36). Our study in accordance with previous ones, have showed inhibition of apelin signaling and reduced plasma concentrations as a potential response to undernutrition (37). Recent studies have highlighted the paramount importance of apelin and its receptor, since they have been proposed as a valuable new treatment target in type 2 diabetes (38, 39).

Our study showed that in both FGR and non-FGR offspring of calorie restricted mothers, liver thyroid hormones' metabolism is inhibited. Thyroid hormones play a key role to thermoregulation, specifically in norepinephrine (NE) controlled thermogenesis (40). Brown adipose tissue thermogenic activity which is triggered by NE is under triiodothyronine (T_3) control

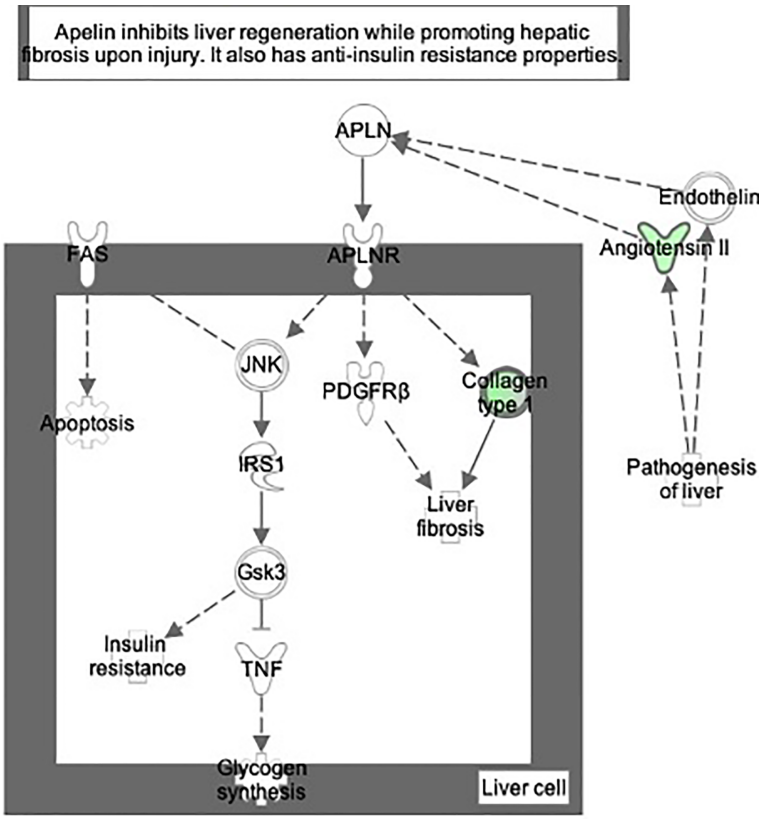

FIGURE 7 | Ingenuity Pathway Analysis of DEPs between FGR vs. Control group. *Inhibition of apelin liver signaling pathway.* **(z = –2.2, p = 8.5e-5)**.

(41). Low T3 plasma levels are associated with impaired thermogenesis and predisposition to diet-induced obesity in neonatal and adult life despite later normalization of T_3 plasma concentrations (42, 43). Hypothermia and transiently low thyroxine levels are common neonatal complications of FGR however no information is available in appropriately grown neonates born to undernourished mothers (44).

Our model suggests inhibition of fatty acid metabolism not only in FGR liver but in non-FGR liver as well. Liver is the central organ of fatty acid metabolism. Both obesity and insulin resistance are closely related with disrupted fatty acid metabolism (26). Inhibition of this metabolic process leads to non-alcoholic fatty liver, liver steatosis, and subsequent insulin resistance deterioration. In a previous study of our team where NEFA (Non-Esterified Fatty Acids) concentrations were compared between FGR and non-FGR rats at one year of age there was no statistical difference between groups. It seems that food restriction produces the same adipose tissue response in both the FGR and non-FGR groups, suggesting that it is the adverse prenatal event that determines certain metabolic profiles rather than birthweight (45).

During the last few decades, a remarkable increase in the prevalence of non-alcoholic fatty liver (NAFLD) in modern western world has been observed (8). Various risk factors namely obesity, insulin resistance or overt diabetes, dyslipidemia, and metabolic syndrome are potential precursors of NAFLD. As non-alcoholic fatty liver seems to be the major cause of chronic liver disease, it is important to recognize individuals with increased risk for this condition, such as FGR offspring, in order to intervene early and prevent its pathogenesis. Recent studies have revealed that NAFLD affects low birth weight offspring even during childhood (46, 47). Although increased hepatic lipids promote insulin resistance, the exact mechanism through which early insulin resistance accelerates the development of non-alcoholic fatty liver in FGR individuals remains unclear (28, 48, 49).

To date, few studies have investigated the proteomic profile of the placenta of FGR human offspring. Bioinformatic analysis of differentially expressed proteins of FGR placentas revealed a distinct proteomic network associated with growth restriction. Most of these studies showed upregulation of proteins related to oxidative stress, cellular apoptosis, inflammation, and intracellular lipid metabolism (50). Moreover, Chassen et al. demonstrated increased expression of two fatty acid transport proteins and seven long chain fatty acids in the cellular triglyceride fraction in placentas of FGR fetuses compared to appropriately grown. These results establish some additional adaptive mechanisms of growth restricted fetuses in order to survive in this adverse intrauterine environment (51).

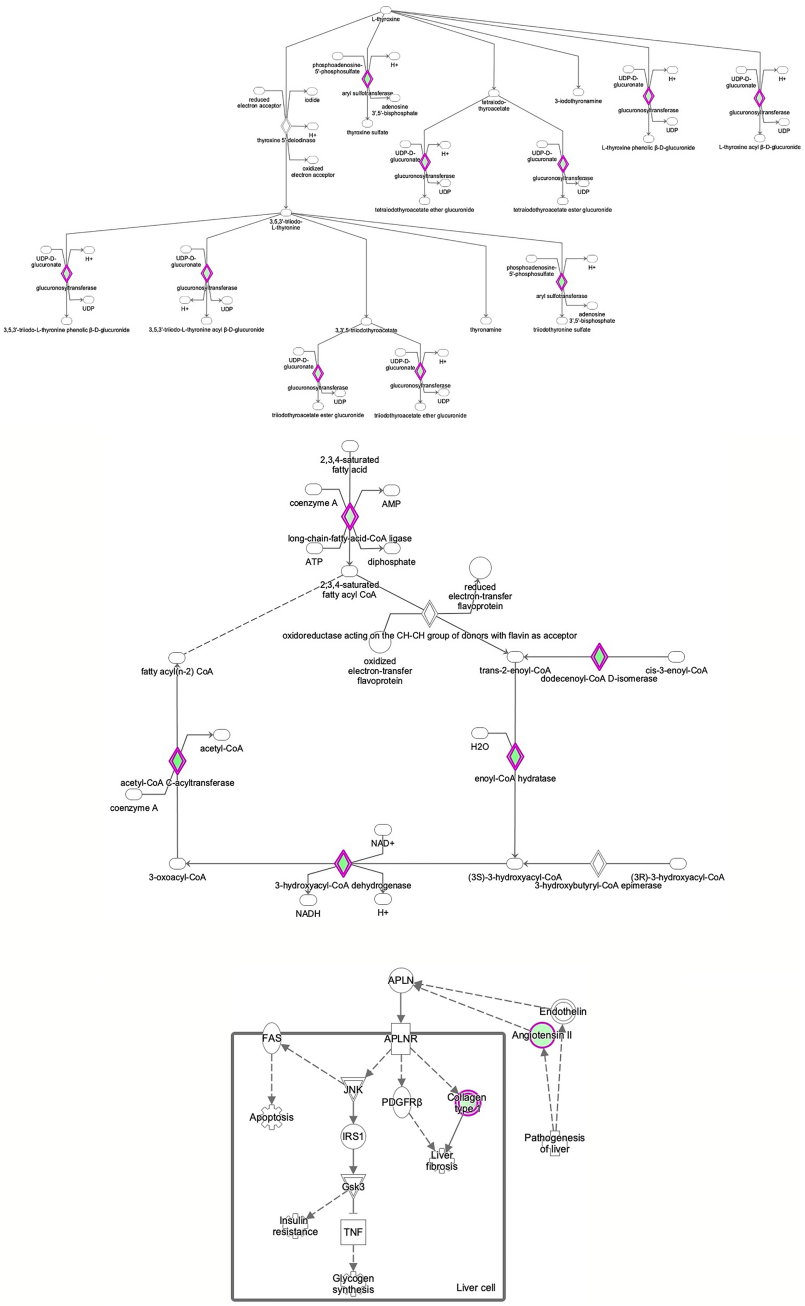

FIGURE 8 | Ingenuity Pathway Analysis of differentially expressed proteins in non-FGR compared to control group showed *inhibition of thyroid hormone metabolism* **(z= −2.0, p = 2.5e-2)**, *fatty acid beta oxidation* **(z = −2.0, p = 1.6e-2)** *and apelin liver signaling pathway* **(z = −2.0, p = 6.7e-3)**. Apelin inhibits liver regeneration while promoting hepatic fibrosis upon injury. It also has an anti-insulin resistance properties.

CONCLUSION

According to fetal programming theory, fetal malnutrition induces adaptive processes that permanently change growth, physiology, and metabolism of the offspring. Maternal undernutrition alters the proteomic profile of the neonatal liver which is a key organ of many metabolic processes supporting homeostasis. In our study, FGR (representing a model of human neonates with growth restriction) and non-FGR pups (representing a model of human infants having experienced adverse intrauterine conditions but born with normal body weight) have developed both common and different metabolic phenotypes. Thus, suggesting that both intrauterine adversities and birthweight determine the metabolic profile of the offspring. This study contributes to a better

understanding of the proteomic mechanisms of liver developmental dysfunction induced by prenatal food restriction and helps to explain the intrauterine origin of adult metabolic disease. Research in both animal and humans should focus on early detection of possible pregnancy complications and adequate prevention and intervention strategies as well, in order to promote postnatal health and ameliorate diseases with developmental origins, such as non-communicable diseases.

STUDY LIMITATION

The extrapolation of our results to human population should be made with caution as in all experimental studies. The use of specific animal model of prenatal malnutrition, the number of animals in each experimental group that should be kept to the minimum and differences between human physiology and disease that are not adequately captured by animal models may limit the strength of our findings.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. All proteomic data are uploaded at the ProteomeXchange Consortium via the PRIDE partner repository (dataset identifier PXD011406) http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD011406.

ETHICS STATEMENT

This animal study was approved by the Ethics Committee of Aretaieion University Hospital, Medical School of the National and Kapodistrian University of Athens with registration number: B 207/13-10-2016 and the Directorate of Veterinary Services (protocol number: 1211/19-03-2018).

AUTHOR CONTRIBUTIONS

Conceptualization, ME; formal analysis, P-MS, AM, AP, AZ, and EE; funding acquisition, P-MS and ME; investigation, P-MS, AM, AP, AZ, and ME; methodology, P-MS, AM, SDG, and ME; project administration, ME; resources, ME and SDG; supervision, KP, PP, NV, SDG, and ME; visualization, P-MS and AM; writing—originaldraft, P-MS and AM; writing— review and editing, AM, KP, ED, NV, PP, SDG, and ME. 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/fendo.2021.684220/full#supplementary-material

Supplementary Table 1 | All identified proteins (peptide FDR p<0.05) by the proteomic analysis.

Supplementary Table 2 | Differentially expressed proteins (DEPs) in FGR vs. control.

Supplementary Table 3 | Differentially expressed proteins (DEPs) in non-FGR vs. control.

Supplementary Table 4 | Commonly up- or down-regulated proteins in both FGR and non-FGR vs. controls.

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Conflict of Interest: SG is Founder, President, and CEO of Proteas Bioanalytics Inc., BioLabs at the Lundquist Institute, 1124 West Carson Street, MRL Building, 3rd Floor, Torrance, CA 90502.

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

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Association Between Paternal Age and Birth Weight in Preterm and Full-Term Birth: A Retrospective Study

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Purpose: While it is well documented that maternal adverse exposures contribute to a series defects on offspring health according to the Developmental Origins of Health and Disease (DOHaD) theory, paternal evidence is still insufficient. Advanced paternal age is associated with multiple metabolism and psychiatric disorders. Birth weight is the most direct marker to evaluate fetal growth. Therefore, we designed this study to explore the association between paternal age and birth weight among infants born at term and preterm (<37 weeks gestation).

Methods: A large retrospective study was conducted using population-based hospital data from January 2015 to December 2019 that included 69,964 cases of singleton infant births with complete paternal age data. The primary outcome was infant birth weight stratified by sex and gestational age including small for gestational age (SGA, 10th percentile) and large for gestational age (LGA, 90th percentile). Birth weight percentiles by gestational age were based on those published in the INTERGROWTH-21st neonatal weight-for gestational-age standard. Logistic regression analysis and linear regression model were used to estimate the association between paternal age and infant birth weight.

Results: Advanced paternal age was associated with a higher risk for a preterm birth [35–44 years: adjusted odds ratio (OR) = 1.13, 95%CI (1.03 to 1.24); >44 years: OR = 1.36, 95%CI (1.09 to 1.70)]. Paternal age exerted an opposite effect on birth weight with an increased risk of SGA among preterm infants (35–44years: OR = 1.85, 95%CI (1.18 to 2.89) and a decreased risk among term infant (35–44years: OR = 0.81, 95%CI (0.68 to 0.98); >44 years: OR = 0.50, 95%CI (0.26 to 0.94). U-shaped associations were found in that LGA risk among term infants was higher in both younger (<25 years) (OR = 1.32; 95% CI, 1.07 to 1.62) and older (35–44 years) (OR = 1.07; 95% CI, 1.01 to 1.14) fathers in comparison to those who were 25 to 34 years old at the time of delivery.

Conclusions: Our study found advanced paternal age increased the risk of SGA among preterm infants and for LGA among term infants. These findings likely reflect a

pathophysiology etiology and have important preconception care implications and suggest the need for antenatal monitoring.

Keywords: paternal age at birth, birth weight, large for gestational age, obesity, small for gestation age

INTRODUCTION

While it is well documented that advanced age among women is an important risk factor for infertility, miscarriage, and offspring genetic defects (1), less is known about the effects of advanced age on reproductive impairment among men. Globally, paternal age at childbirth is steadily increasing. In 1993, the proportion of fathers aged 35 to 54 in the UK was 25%, and in 2003 this proportion rose to 40% (2). From 1972 to 2015, the National Vital Statistics System data has shown that the mean paternal age has increased from 27.4 to 30.9 years in US (3). In 2014, the Chinese government started to implement an exemption to the only-child policy allowing a second child which contributed to the phenomenon that many older couples deciding to have a second child. Researchers have shown that the risk increase in incontrovertible paternal age-related adverse conditions starts around 35 years old of paternal age (4). There is preliminary evidence to suggest that advanced paternal age may increase the occurrence of adverse pregnancy outcomes such as miscarriage (5), stillbirth (6) or preterm birth (7). The adverse offspring outcomes include higher rates of congenital malformations (8), malignancies (9), early onset schizophrenia (10), autism (11) and other psychiatry or academic morbidities (12).

Birth weight is often considered to be the most direct marker to evaluate fetal growth in utero and intrauterine environment quality. The Developmental Origins of Health and Disease Science clearly suggests that early life growth and development influences health and well-being trajectories into adulthood (13). It has been suggested that advanced paternal age may contribute to the increasing rate of low birth weight infants which may indirectly result in a higher incidence of infant mortality, childhood morbidity (14) and cardiovascular disease in adulthood (15). Because there is an increased risk of preterm birth and very early preterm birth among offspring of men with advanced paternal age, low birth weight may not be an ideal outcome to characterize the effect of advanced paternal age on infant birth weight. Thus, we used neonatal weight for gestational age standard as a more precise evaluation method and conducted this large retrospective study to explore the association between advanced paternal age and birth weight.

MATERIALS AND METHODS

Study Population

This study was performed at the International Peace Maternity and Child Health Hospital located in Shanghai, China. As a hospital delivery population-based cohort study, female participants were those who underwent first-trimester prenatal screenings at the hospital between January 2015 and December 2019. A total of 80,811 women registered at the obstetrics department. After excluding 2,332 cases of twins and 8,803 cases of missing the data of paternal age, 69,964 cases were included in this study (**Figure 1**). All of them had complete data of maternal age, paternal age, gestational week and birth weight. All data are available at the ResMan Manager of Chinese Clinical Trial Registry (www.medresman.org) with the registration number ChiCTR2000038345. Ethics approval for this study was provided by the participating hospital medical ethical committee (no.GKLW2013-9151) and all participants provided written informed consent to have their data included in retrospective analyses.

Data Collection

All data were collected by hospital nurses, medical residents, and gynecologists with the use of electronic medical record. Parental age, education level and demographic data were collected *via* first clinical presentation. Maternal height and weight were measured to calculate early pregnancy body mass index (BMI) at the first prenatal visit. BMI was used to characterize the women as underweight (BMI <18.5 kg/m^2), normal (BMI 18.5 to 24.9 kg/m^2), and overweight or obesity (OWO) (BMI ≥25 kg/m^2) using the World Health Organization criteria (16). Gestational age was calculated based on the date of last menstrual period and adjusted by ultrasonography in early pregnancy. Hypertension and diabetes mellitus whether preexisting or induced by pregnancy were prospectively recorded in the medical records. Pregnancy-induced hypertension was defined as a newly onset hypertension without proteinuria with a blood pressure higher than 140/90 mmHg after 20 weeks' gestation (17). Gestational diabetes mellitus was defined according to the diagnostic standard of the American Diabetes Association (18). Preterm birth (PTB) was defined as birth before 37 weeks of gestation.

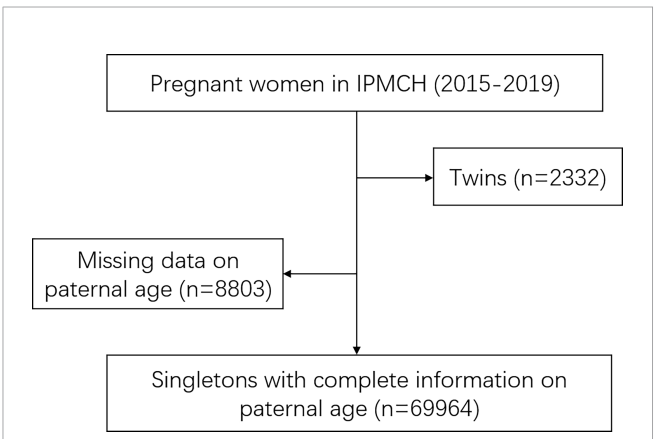

FIGURE 1 | Flow chart: 69,964 cases are enrolled in this study.

Very early preterm birth (VPTB) was defined as birth before 34 weeks of gestation. Late preterm birth was defined as birth during 34 to 36 weeks of gestation.

The primary outcome of our study was birth weight. Small for gestational age (SGA) was defined as a birth weight less than 10th percentile for gestational age and large for gestation age (LGA) was defined as a birth weight more than 90th percentile for gestational age. The percentiles were based on those published in the INTERGROWTH-21st neonatal weight-for gestational-age standard (19). Information of birth weight, fetal sex and gestational age were recorded on electronic medical files.

Statistical analysis

We use a multiple logistic regression model to analyze the association of paternal age and multiple birth weight and pregnancy outcomes. Parental age was defined as the father's age at the time of childbirth. We analyzed parental age as continuous variables and estimated the risk for SGA and LGA associated with a 10-year increase in age. We first used restricted cubic splines with five knots at the fifth, 27.5th, 50th,72.5th and 95th percentiles (20) to assess the potential nonlinear response for continuous age variables and risks of SGA, LGA, LBW and Macrosomia. For consistency with previous studies (7), paternal age variables were grouped into 10-year intervals and categorized into the following four categories: younger than 25 years, 25 to 34 years, 35 to 44 years, and 45 years or older. The reference group was set as 25 to 34 years for categorical age analyses. Continuous variables were described as medians with 95% CI. Categorical variables were represented as frequencies with proportions. Linear regression coefficients and logistic regression odds ratios with 95% confidence intervals were used to estimate pregnancy and infant outcomes in relation to paternal age levels (<25, 25 to 34, 35 to 44, and >44). Potential confounders were considered in multivariable analysis. Covariates, including maternal age (<25, 25 to 34, or >35 years), maternal education years (<9, 10 to 12, 13 to 15, or >16 years), marital status (live with or without fathers), maternal ethnicity (Han or others), maternal BMI (underweight, normal, overweight or obesity), were included to estimate the associations between paternal age and risk for adverse birth weight outcome. Subgroups of women with different age (<24, 25 to 34, or >35 years) and BMI ranges (BMI <18.5 kg/m^2, 18.5 to 24.9 kg/m^2, and >25 kg/m^2) are generated for sensitivity analysis. Paternal age variables which grouped into 5-year intervals are also analyzed for sensitivity analysis and categorized into the following eight categories: younger than 25 years, 25 to 29 years, 30 to 34 years, 35 to 39 years, 40 to 44 years, 45 to 49 years, 50 to 54 years and 55 years or older. Further, we studied the combined effects of maternal BMI and paternal age on birth weight by adding a product interaction term of the maternal BMI × paternal age to the models. The same analysis is also conducted on the combining effects on the occurrence of SGA and LGA. A heat map was constructed to display the differences (red indicates high risk, blue indicates low risk).

For variables with missing data, multiple imputations according to the Markov chain Monte Carlo method were used (21). There were 0.75% cases missing data of maternal BMI. Missing data were imputed using multiple imputation by chained equations with predictive mean matching. Five imputed data sets were generated for analyses. No significant differences in descriptive characteristics were found between the original and imputed data sets. All statistical analyses were performed using R statistical software version 4.0.3 (package rms, visreg), Statistical Package of Social Sciences version 25.0 for Windows (IBM Corp, Armonk, NY) or GraphPad Prism 8.0.1.

RESULTS

Population Characteristics

In our analysis, 69,964 cases were included where data files were organized sequentially by year and all available demographic variables, including age, ethnicity, education level, marital status, maternal BMI in early pregnancy, etcetera were extracted. Population characteristics are presented (**Table S1**). The median (95%CI) paternal age was 32.5 (26.0 to 41.0) years with maternal age decreasing slightly to 30.1 (25.0 to 38.0) years. The median (95%CI) birth weight was 3,329.0 (2,640.0 to 4,025.0) g. The study population was primarily nulliparous (71.8%) with a naturally conceived pregnancy (94.6%) and not diagnosed with gestational diabetes (86.3%) or pregnancy-induced hypertension (95.9%). The majority of women (87.4%) had an upper secondary-level education or a bachelor's degree. Paternal age was categorized into 10-year intervals: <25 (n = 752; 10.7%), 25–34 (n = 48,545; 69.4%), 35–44 (n = 19,263; 27.5%) and 45 or older (n = 1,404; 2.0%). The overall rate of SGA and LGA was 1.8 and 17.6% respectively.

Paternal Age and the Risk of Adverse Birth Weight and Preterm Birth

Stratified by the four paternal age groups, the occurrence of adverse pregnancy outcomes, including PTB and VPTB as well as gestational complications including gestational diabetes mellitus and pregnancy-induced hypertension, were significantly increased with by aging (**Table 1**). For infant outcomes, a higher risk for a lower Apgar score (<8) and adverse fetal composite (including stillbirth, birth before 28 weeks and SGA less than three percentile) was observed for father older than 35 in comparison to those aged 25–34. As for birth weight, incidence for LGA positively associated with paternal age while SGA was negatively associated. Using linear regression modeling to study the association between paternal age and multiple birth weight outcomes, there is an observational U-shaped nonlinear association between LGA (P <.001) and LBW (P <.001) with paternal age (**Figures 2A–D**). Further, gestational age was lower among infants born to fathers aged 35–44 by an average of 0.11 weeks (95% CI, −0.14 to −0.08 weeks) and fathers with advanced age were 13% more likely to have a PTB compared with younger fathers (OR, 1.13; 95%CI, 1.03 to 1.24, **Table 2**). Similarly, fathers who were 45 years of age and older had infant born 0.15 weeks younger (95%CI, −0.23 to −0.06) and were 36% more likely to have a PTB compared with younger fathers (OR, 1.36; 95%CI, 1.09 to 1.70). Partners of fathers with advanced age also had increased rates of gestational diabetes and pregnancy induced hypertension although this is not reach statistical significance.

TABLE 1 | Maternal characteristics, pregnancy and infant outcomes by paternal age group.

	Paternal age (years)			
Maternal characteristics	<25 (n = 752)	25–34 (n = 48,545)	35–44 (n = 19,263)	>44 (n = 1,404)
Maternal age (mean, 95%CI)	24.0 (22.00–29.00)	29.0 (25.0–34.0)	35.0 (29.0–40.0)	37.0 (29.0–43.0)
Maternal BMI (mean, 95%CI)	19.9 (16.70–25.94)	20.6 (17.4–26.0)	21.3 (17.9–26.9)	21.3 (18.0–26.7)
Pregnancy outcomes				
Gestational weeks (mean, 95%CI)	39.30 (36.8–40.0)	39.20 (37.0–41.0)	38.60 (36.4–40.5)	38.60 (36.2–40.4)
Preterm birth (n, %)	37 (4.9)	2,328 (4.8)	1,226 (6.4)	110 (7.8)
Very early preterm birth (n, %)	7 (0.9)	382 (0.8)	274 (1.4)	26 (1.9)
Gestational diabetes (n, %)	58 (7.7)	5,693 (11.7)	3,523 (18.3)	321 (22.9)
Gestational hypertension (n, %)	27 (3.6)	1,806 (3.7)	926 (4.8)	87 (6.2)
Infant outcomes				
Birth weight (mean, 95%CI)	3,340 (2,641.5–4,057.0)	3,330 (2,650.0–4,020.0)	3,345 (2,610–4,035)	3,311 (2,496.2–4,057.5)
Low birth weight (n, %)	25 (3.3)	1,315 (2.7)	676 (3.5)	70 (4.9)
Macrosomia (>4,000 g)	46 (6.1)	2,588 (5.3)	1,115 (5.8)	86 (6.1)
Small for gestational age (10%), (n, %)	20 (2.7)	918 (1.9)	304 (1.6)	18 (1.3)
Large for gestational age (90%), (n, %)	127 (16.9)	7,939 (16.4)	3,947 (20.5)	296 (21.0)
Low 5-minute Apgar score (<8), (n, %)	23 (3.1)	1,374 (2.8)	682 (3.5)	55 (3.9)
Adverse fetal composite, (n, %)	1 (0.1)	191 (0.4)	82 (0.4)	6 (0.4)

Values are numbers (percentages) unless stated otherwise. Adverse fetal composite: stillbirth, delivery earlier than 28 weeks, birth weight less than the third percentile for gestational age and sex.

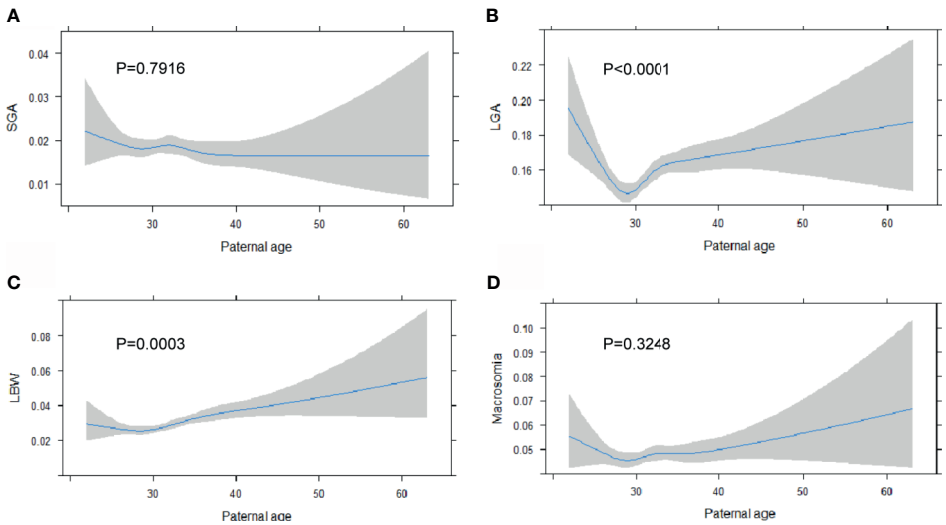

FIGURE 2 | Paternal age is associated with higher risk of adverse birth weight outcomes. Linear regression model is generated for paternal age at birth with the incidence of SGA **(A)**, LGA **(B)**, LBW **(C)** and Macrosomia **(D)**, expressed as predicted prevalence with 95% CIs. Analyses were adjusted for maternal age, BMI, ethnicity, education level and marital status.

Lastly, infants born to fathers aged 34–44 were 23% more likely to be LBW than those born to younger fathers (OR, 1.23; 95% CI, 1.08 to 1.39) and were 7% more likely to be LGA (OR, 1.07; 95%CI, 1.01 to 1.13). There was no significant difference in SGA between the four paternal age groups (**Table 2**).

The Association Between Paternal Age and Birth Weight Differ in Preterm and Term Birth

Given preterm birth and LGA rates increased with paternal age but not the incidence of SGA, we examined if there may be differences in risk for SGA and LGA among infants born term and preterm. As shown in **Figure 3**, paternal age exhibited dichotomous effect on birth weight with increased risk of SGA (35–44years: OR = 1.85, 95%CI (1.18 to 2.89) among infants born preterm birth and a decreased risk for infants born at term (35–44years: OR, 0.81, 95%CI (0.68 to 0.97); >44years: OR, 0.50, 95%CI (0.26 to 0.94). U-shaped associations were found where LGA risk was higher among young fathers (<25 years) (OR, 1.32; 95% CI, 1.07 to 1.62) and those who are older (35–44 years) (OR, 1.07; 95% CI, 1.01 to 1.14) in comparison to infant born among fathers who were 25 to 34 years old at delivery (**Table S2**). This result is consistency with the outcomes when stratified paternal age in a 5-year interval (**Table S3**). When analyzing paternal age

TABLE 2 | Unadjusted and adjusted risk of major outcomes stratified by paternal age in 69,964 pregnancies.

	Paternal age (years)			
Unadjusted model, OR (95% CI)	<25 (n = 752)	25–34 (n = 48,545)	35–44 (n = 19,263)	>44 (n = 1,404)
Gestational weeks (weeks)	0.13 (0.03 to 0.23)	reference	−0.31 (−0.34 to −0.29)	−0.44 (−0.52 to −0.37)
Preterm birth (<37 **w**)	1.03 (0.74 to 1.43)	reference	1.35 (1.26 to 1.45)	1.69 (1.38 to 2.06)
Very early preterm birth (<34 **w**)	1.19 (0.56 to 2.51)	reference	1.82 (1.56 to 2.13)	2.38 (1.59 to 3.55)
Birth weight (mean, 95%CI)	3.17 (-28.69 to 35.04)	reference	3.01 (−4.37 to 10.40)	−17.39 (−40.88 to 6.08)
Low birth weight (<2,500 g)	1.24 (0.83 to 1.85)	reference	1.31 (1.19 to 1.44)	1.89 (1.47 to 2.41)
Small for gestational age (10%)	1.42 (0.91 to 2.22)	reference	0.83 (0.73 to 0.95)	0.67 (0.42 to 1.08)
Large for gestational age (90%)	1.04 (0.86 to 1.26)	reference	1.32 (1.26 to 1.38)	1.37 (1.20 to 1.56)
Macrosomia (>4,000 g)	1.16 (0.86 to 1.56)	reference	1.09 (1.02 to 1.17)	1.16 (0.93 to 1.45)
Low 5-minute Apgar score (<8)	1.08 (0.71 to 1.65)	reference	1.26 (1.15 to 1.38)	1.40 (1.06 to 1.84)
Adverse fetal composite, (n, %)	0.34 (0.05 to 2.41)	reference	1.08 (0.84 to 1.40)	1.09 (0.48 to 2.45)
Adjusted model, OR (95% CI)				
Gestational weeks (weeks)	−0.40 (−0.14 to 0.07)	reference	−0.11 (−0.14 to −0.08)	−0.15 (−0.23 to −0.06)
Preterm birth (<37 **w**)	1.21 (0.86 to 1.70)	reference	1.13 (1.03 to 1.24)	1.36 (1.09 to 1.70)
Very early preterm birth (<34 **w**)	1.63 (0.76 to 3.48)	reference	1.37 (1.12 to 1.69)	1.55 (0.98 to 2.47)
Birth weight (mean, 95%CI)	13.11 (−19.51 to 45.72)	reference	−5.83 (−15.27 to 3.60)	−14.34 (−39.70 to 11.02)
Low birth weight (<2,500 g)	1.32 (0.87 to 2.01)	reference	1.23 (1.08 to 1.39)	1.65 (1.25 to 2.19)
Small for gestational age (10%)	1.29 (0.81 to 2.07)	reference	0.92 (0.78 to 1.08)	0.69 (0.41 to 1.16)
Large for gestational age (90%)	1.28 (1.05 to 1.57)	reference	1.07 (1.01 to 1.13)	1.10 (0.94 to 1.26)
Macrosomia (>4,000 g)	1.26 (0.92 to 1.72)	reference	1.01 (0.91 to 1.11)	1.14 (0.90 to 1.46)
Low 5-minute Apgar score (<8)	1.24 (0.68 to 2.28)	reference	1.14 (0.96 to 1.35)	1.17 (0.75 to 1.80)
Adverse fetal composite, (n, %)	0.345 (0.05 to 2.49)	reference	1.241 (0.88 to 1.74)	1.113 (0.43 to 2.85)

Analyses were adjusted for maternal age, BMI, ethnicity, education level and marital status. Values are linear regression coefficients or logistic regression odds ratios with 95% confidence intervals. Adverse fetal composite: stillbirth, delivery earlier than 28 weeks, birth weight less than the third percentile for gestational age and sex.

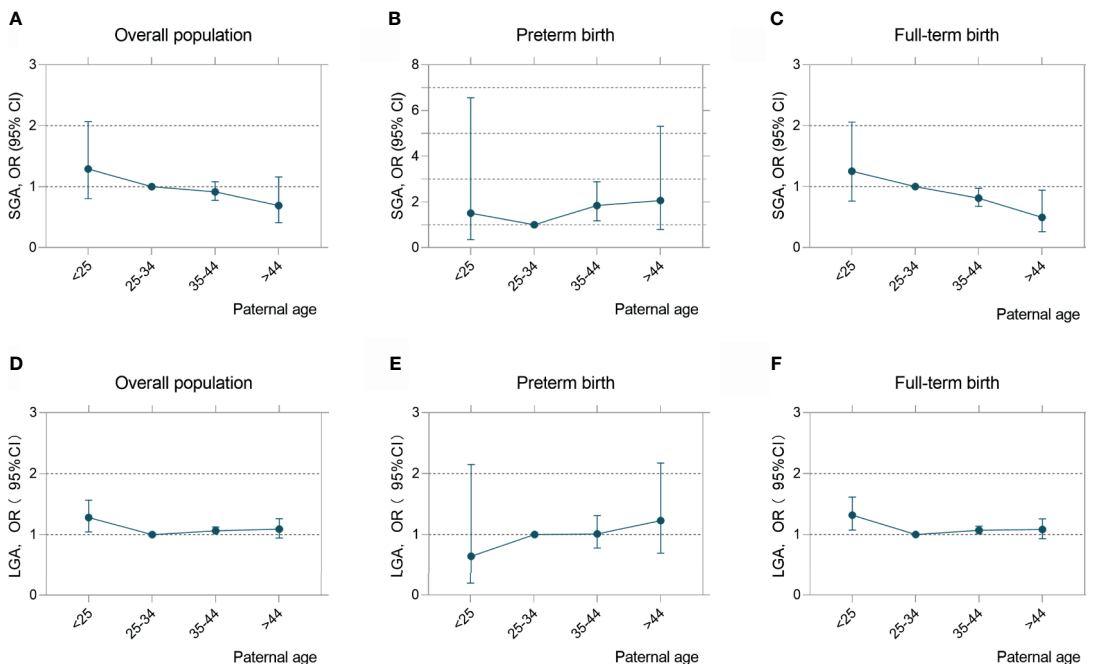

FIGURE 3 | Paternal age exerted an opposite effect on birthweight among preterm and term infants. Adjusted odds ratios (ORs) and 95% confidence intervals for risks of SGA **(A–C)** and LGA **(D–F)** according to paternal age in overall population, preterm birth and full-term birth infants. Analyses were adjusted for maternal age, BMI, ethnicity, education level and marital status.

as a continuous variable and examining the linear association between SGA and LGA rates by the three gestational age subgroups (**Table 3**), there was no increased risk for SGA or LGA among VPTB infants. However, per-year increases in paternal age were associated with an increased risk for SGA among late preterm birth infants (adjusted OR, 1.07; 95% CI, 1.01 to 1.13) and a decreased risk among full-term infants (adjusted OR, 0.99; 95%CI, 0.97 to 1.00). A per-unit increase

TABLE 3 | Per-Unit Increase of paternal age and Risks of SGA and LGA by preterm and full-term birth group.

	Very early preterm birth: <34 w (n = 688)	Late preterm birth: 34–36w (n = 3,017)	Full-term birth: ≥37 w (n = 66,259)
SGA			
n (%)	78 (11.30)	71 (2.40)	1,111 (1.70)
OR (95%CI)	1.01 (0.96 to 1.05)	1.03 (0.98 to 1.07)	0.97 (0.96 to 0.98)
Adjusted OR (95%CI)	0.96 (0.90 to 1.03)	1.07 (1.01 to 1.13)	0.99 (0.97 to 1.00)
LGA			
n (%)	142 (20.60)	412 (13.70)	11,755 (17.70)
OR (95%CI)	0.99 (0.96 to 1.03)	1.01 (0.99 to 1.03)	1.03 (1.03 to 1.04)
Adjusted OR (95%CI)	0.99 (0.94 to 1.05)	1.00 (0.96 to 1.03)	1.01 (1.01 to 1.02)

Adjusted for maternal age, BMI, ethnicity, education level and marital status; per-unit increase are considered as one year increase in paternal age.

in paternal age was also associated with an increased risk for LGA among full-term infants but not preterm infants (adjusted OR, 1.01; 95% CI, 1.01 to 1.02).

Combined Effects of Paternal Age and Maternal BMI on the Outcome of Birth Weight

Maternal BMI in early pregnancy have a crucial impact on the birth weight outcome. From the results we describe above, we hypothesized that paternal age has greater effects on birth weight as well as the incidence of SGA and LGA. Therefore, we studied the combined effects of two factors. Standardized birth weights adjusted by gestational age are generated for the next analysis. A heatmap (filled contour plot) for the combined association of maternal BMI (y-axis) and paternal age (x-axis) with birth weight (SD) (z-axis; red indicates higher birth weight, blue indicates lower birth weight) was plotted (**Figure 4**). We generate the heatmap of overall population and three subgroups categorized by maternal BMI. First, in overall populations, the interaction between paternal age and maternal BMI was significant for the birth weight for gestational age (P = 0.032). When stratified by maternal BMI, the interaction contribution of SGA did not reach significant difference (**Figure S4**). Conversely, the effect estimates for paternal age differ in LGA by stratifications. Considerable combing effects of two factors on higher risk of LGA in two subgroups are also identified (BMI: 18.5–24.9, P for interaction = 0.015; BMI >24.9, P for interaction = 0.009, **Figures 4C, D**).

Sensitivity Analysis

To further investigate the association between paternal age and birth weight, stratification by multiple maternal factors were analyzed. First, after stratification by maternal age, paternal age remained significantly associated with SGA and LGA (**Tables S4**, **S5**). When maternal age was between 25 and 34 years, the adjusted odds ratio of older than 45 years was 1.48 with 95% CI, 1.14 to 1.91 compared to the reference group in LGA (**Figure S3**). When maternal BMI was in the normal range (18.5–24.9 kg/m^2), fathers aged 35–44 years had a 9% (OR, 1.09; 95%CI, 1.02 to 1.16) and those aged >45 years had a 15% (OR, 1.15; 95%CI, 0.98

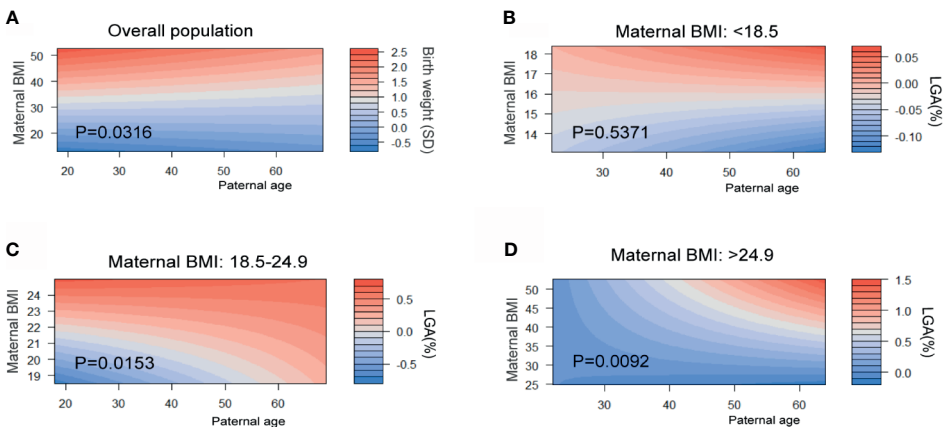

FIGURE 4 | Combined effects of maternal BMI in early pregnancy and paternal age on birth weight and LGA. Maternal BMI were categorized into three stages: underweight (BMI <18.5 kg/m^2), normal (BMI 18.5 to 24.9 kg/m^2), and overweight or obesity (OWO) (BMI ≥25 kg/m^2). Heat map (filled contour plot) for the correlation of gestational age–adjusted birth weight **(A)** and paternal age in overall population (red indicates increased gestational age–adjusted birth weight; blue indicates decreased gestational age–adjusted birth weight). Heat map for the prevalence of LGA in three maternal BMI levels **(B–D)** (red indicates high risk, blue indicates low risk) according to their interaction with maternal BMI and paternal age are generated. Analyses were adjusted for maternal age, BMI, ethnicity, education level and marital status.

to 1.35) increased likelihood of having a LGA infant in comparison to younger fathers (**Figure S4**). Similarly, the risk for SGA decreased with increasing paternal age with exception when mother was in obesity or overweight status (**Figure S4**). When categorized by IVF and parity, the same trend was observed among the non-IVF and nulliparous populations while in the group of women with IVF treatment and multiple parity a significant difference was not found. Similar sensitivity analysis was also conducted in paternal age grouped in a 5-year interval (**Tables S6**, **S7**).

DISCUSSION

Main Findings

In this large retrospective population-based cohort study examining over 69,000 hospital deliveries we found paternal age has a significant effect on birth weight with advanced paternal age increasing the risk of SGA among preterm infants and decreasing the risk among infants born at term. U-shaped associations were found in that LGA risk was higher among father <25 years and those who are older between 35 and 44 years of age especially among term infants when compared to infants whose fathers were aged 25 to 34 years of age.

We studied the association between paternal age and birth weight in multiple linear regression model and observed a strong U-shape association between paternal age and LGA in the overall cohort population. Due to the coexisting risk of LGA and LBW, we considered that high preterm birth risks may be a possible confounding factor when examining the association between paternal age and birth weight. When we categorized the population gestational weeks to observe the potential different impact of paternal age on birth weight, paternal age demonstrated dichotomous effect on birth weight with an increased risk of SGA among preterm and late preterm infants and a decreased risk among full-term infants. LGA risk was significantly higher among both younger (<25 years) and older (35–44 years) fathers when compared to infants whose fathers were aged 25 to 34 years in the overall population and the full-term subgroup. Further, maternal BMI and paternal effect has a combined effect on the prevalence of LGA. These results suggest the effect of advanced paternal age on birth weight is complicated and should be evaluated according to clinical circumstances.

Interpretations

A significant number of studies have examined the association between maternal characteristics and child outcomes while few have examined paternal factors. Advanced paternal age has been reported to increase the risk for adverse pregnancy and neonatal outcomes such as low birth weight and preterm birth (7). It also may exert a long-term negative impact on the child into adulthood including metabolic risks (22) and psychological disease (11, 12). Preliminary evidence also suggests aging fathers may enhance the mortality (9) and shorten the life expectancy of their child (23). One pathway may be related to research indicating paternal age may be associated with adverse lipid profiles (24) and possibly an elevated risk for obesity in young adulthood (22). Neonatal birth weight as a crucial marker to evaluate fetal growth and reflects an intrauterine environment which can have a significant effect on adulthood heath. Large gestation age (LGA) is as an early-life trait of adulthood obesity and diabetes (25). However, few studies have been focused on studying the association between paternal age and birth weight adjusted by gestational age. Our study addresses an important clinical gap between paternal factors and child outcomes by clearly outlining a positive association between advanced paternal age and LGA among term infants. Besides, small gestation age (SGA) is a crucial indicator of infant congenital abnormality (8) and mortality (26, 27). Researchers also found that preterm children born as SGA also had a higher risk of obesity when comparing to those born as appropriate for gestational age in childhood (28). This interesting phenomenon can be explained by rapid compensational weight gaining in infancy (29, 30). Those born SGA and develop high BMI by catch-up growth in childhood are at increased risk of abnormal glucose metabolism in adulthood (31).

Maternal age and BMI are two determinant factors for the development of LGA (32). As such, we divided 69,964 cases into subgroups stratified by maternal age and maternal BMI individually in order to examine whether the association between paternal age and birth weight is stable. We found that when maternal age is in a normal range (25–34), the infants of aging fathers are at a higher risk for LGA. A significantly elevated risks for LGA contributed by advanced paternal age was confirmed among a subgroup of normal-BMI women. However, we did not observe significance difference in GDM risk among four paternal age groups though GDM is considered as a crucial complication contribute to a higher risk of LGA (33).These results strengthened the independent effect of advanced paternal age on LGA.

When considering the underlying mechanism of this phenomenon, previous research involving mouse models suggests that advanced paternal age could exert adverse metabolic effects on offspring by impairing glucose tolerance (34). One possible explanation is that aging exerts epigenetic changes in male sperm including methylation status and histone modifications which may be an explanation for its effect on an offspring's increased risk for metabolism health conditions (23).

Strength and Limitations

The major strength of the current study is the rigorous examination of the association between advanced paternal age and birth weight for gestational age for the first time. Complete medical information allowed us to study maternal confounding factors such as age, educational level, marital status and early pregnancy BMI. However, several limitations should be noted. First, this study was a hospital-based cohort, and while the homogeneous ethnicity of the cohort increased the internal validity it decreased the external validity. Second, due to the limited paternal demographic data, our conclusion requires future studies to exclude paternal confounding factors other than age. Third, among the population of non-nullipara

women, comparing the pregnancy and fetal outcome within family can minimized the unobserved parental characteristics (35). A sibling fixed model can assist in confirming our results.

In conclusion, our results suggest that paternal age is associated with birth weight. Paternal age had a significant effect on infant birth weight with an increased risk for SGA among preterm infants and LGA among term infants. These findings likely reflect a pathophysiology pathway and have clinical implications for prenatal counseling and pediatric care. Future studies including prospective cohorts or randomized controlled trials are required to confirm the developmental effect of advanced paternal age on child in order to guide preventative interventions to minimize adverse fetal outcomes. Elucidating the mechanisms of how male aging can influence infant growth trajectories in clinically important given more families are selecting to have children at an advanced age.

DATA AVAILABILITY STATEMENT

All data are available at the ResMan Manager of Chinese Clinical Trial Registry (www.medresman.org) with the registration number ChiCTR2000038345.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the participating hospital medical ethical committee (no.GKLW2013-9151) and all participants provided written informed consent to have their data included in retrospective analyses. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HH and YWu had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Concept and design: YTM, CZ, and YWu. Acquisition, analysis, or interpretation of data: YTM, CZ, YWa, YCM and JS. Drafting of the manuscript: YTM. Revision of the manuscript: YWu and C-LD. Supervision: HH. 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/fendo.2021.706369/full#supplementary-material

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REVIEW
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Adverse Impact of Environmental Chemicals on Developmental Origins of Kidney Disease and Hypertension

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Chronic kidney disease (CKD) and hypertension are becoming a global health challenge, despite developments in pharmacotherapy. Both diseases can begin in early life by so-called "developmental origins of health and disease" (DOHaD). Environmental chemical exposure during pregnancy can affect kidney development, resulting in renal programming. Here, we focus on environmental chemicals that pregnant mothers are likely to be exposed, including dioxins, bisphenol A (BPA), phthalates, per- and polyfluoroalkyl substances (PFAS), polycyclic aromatic hydrocarbons (PAH), heavy metals, and air pollution. We summarize current human evidence and animal models that supports the link between prenatal exposure to environmental chemicals and developmental origins of kidney disease and hypertension, with an emphasis on common mechanisms. These include oxidative stress, renin-angiotensin system, reduced nephron numbers, and aryl hydrocarbon receptor signaling pathway. Urgent action is required to identify toxic chemicals in the environment, avoid harmful chemicals exposure during pregnancy and lactation, and continue to discover other potentially harmful chemicals. Innovation is also needed to identify kidney disease and hypertension in the earliest stage, as well as translating effective reprogramming interventions from animal studies into clinical practice. Toward DOHaD approach, prohibiting toxic chemical exposure and better understanding of underlying mechanisms, we have the potential to reduce global burden of kidney disease and hypertension.

Keywords: chronic kidney disease, hypertension, DOHaD (developmental origins of health and disease), environmental chemical, oxidative stress, endocrine disruption chemical, renin-angiotensin system

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1 INTRODUCTION

The association between maternal exposure to environmental risk factors and the increased risk for developing adult disease has received increasing recognition in recent decades. This phenomenon is referred to as "developmental programming" or "developmental origins of health and disease" (DOHaD) (1, 2). The DOHaD hypothesis gained attention after the emergence of observational studies from the famine cohorts combined with several subsequent epidemiologic investigations (3–5),

illuminating events before birth can predispose offspring towards non-communicable diseases (NCDs) in later life. Considering the increasing burden of global NCDs, therefore, the WHO informed the public about NCD prevention and control policies (6). So much so, in fact, that the DOHaD concept becomes a key prevention strategy to limit the passage of NCD risks to the next generation (7).

Kidney disease and hypertension are highly prevalent NCDs worldwide (8). About 10% of the global population is affected by chronic kidney disease (CKD) (8). Despite hypertension prevalence is highest in older populations, up to 20% of young adults are hypertensive (9). Kidney disease and hypertension have a bidirectional relationship (10), such that CKD is a complication of uncontrolled hypertension and hypertension is a frequent finding in kidney disease. Both kidney disease and hypertension can take their origins in early life (11). During critical period of development, the fetal kidney is particularly vulnerable to adverse impacts of gestational events, leading to functional and structural modifications, known as renal programming (12). A wide range of maternal insults can induce renal programming, giving rise to kidney disease and hypertension in later life. These include maternal malnutrition, maternal illness, substance abuse or medication use during pregnancy, exposure to environmental chemicals, etc (13–16). Numerous studies have reported the adverse renal effects that occur following exposure to a broad spectrum of environmental chemicals (17–20). However, little is known about the long-term adverse consequences on the offspring from maternal exposure to environmental chemicals in pregnancy. Of note, emerging evidence supports a "two-hit" hypothesis that explains the developmental programming of adult diseases (21). Hypertension and kidney disease may develop with two sequential hits: the first hit being the prenatal environmental chemical exposure, followed by the second hit in response to postnatal insult. CKD is characterized by a progressive loss of nephrons. There is a ten-fold variation in nephron number at birth (22), and a further decrease over the life cycle. Reduced nephron number can stimulate hypertrophy of remaining nephrons, resulting in glomerulosclerosis and more nephron loss. From an evolutionary perspective, the transition of hypertrophied nephrons to fibrosis is considered to be maladaptive (23). Accordingly, the recognition of the contribution of environmental chemicals to the changing nephron formation and numbers from embryo through senescence could provide new insight into the prevention of CKD.

In this Review, we focus on environmental chemicals that pregnant mothers are likely to be exposed as a consequence of normal consumer activities, that is, dioxins, bisphenol A (BPA), phthalates, per- and polyfluoroalkyl substances (PFAS), polycyclic aromatic hydrocarbons (PAH), heavy metals, and air pollution. We aim to provide an overview of maternal exposure to environmental chemicals implicated in developmental origins of kidney disease and hypertension. The mechanisms mediating renal programming will be a special focus, and their interrelationships to individual chemicals will be discussed. Furthermore, the potential of preventive approach to protect offspring against developmental origins of kidney disease and hypertension will be summarized. A drawing schematic summarizing the sources of environmental chemicals, adverse impact of maternal exposure on kidney disease and hypertension on adult offspring, and common mechanisms underlying renal programming are depicted in **Figure 1**.

The PubMed/MEDLINE database was searched for English-language and full-text articles published from 1980 to June 2021 using the following search terms: "bisphenol A", "polychlorinated dibenzo-p-dioxins", "dioxins", "polychlorinated biphenyls", "polychlorinated biphenyl", "perfluoroalkyl acid", "perfluoroalkyl", "perfluoroalkyl compound", "phthalates", "phthalic acids", "polycyclic aromatic hydrocarbons", "heavy metal", "lead", "mercury", "cadmium", "air pollution", "particulate matter", "renal function", "kidney", "nephrogenesis", "blood pressure", "albuminuria", "hypertension", "developmental programming", "DOHaD", "mother", "maternal", "pregnancy", "gestation", "offspring", "progeny", and "prenatal". Additional studies were then selected and assessed based on appropriate references in eligible papers.

2 SOURCES AND ADVERSE RENAL EFFECTS OF ENVIRONMENTAL CHEMICALS

Various environmental chemicals pose a broad range of adverse effects on the kidney. **Table 1** illustrates the major source and reported adverse renal effects for environmental chemicals that individuals are likely to be exposed during normal consumer activity. Each of these chemicals will be discussed in turn.

2.1 Dioxins

The chemical name for dioxin is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most extensively studied and toxic dioxin. While the name "dioxins" is habitually used for the family of structurally and chemically related polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzo-p-furans (PCDFs), and dioxin-like polychlorinated biphenyl (PCB). Dioxins are synthetic halogenated aromatic hydrocarbons, emitted mostly from anthropogenic sources like manufacturing of pesticides, bleaching of wood pulp and waste incineration (24) (**Table 1**). The presence of dioxins in the environment and the risk of exposure for human health has raised great concern. The half-lives of PCDDs and PCDFs range from 2–15 years (25); as such, dioxins last a long time in fat tissue of the body. Dioxins tend to accumulate in the food chain in the environment. Accordingly, pregnant mothers can be exposed to these chemicals by eating diet high in animal fat or occupational exposure. A high-level exposure to dioxins is associated with decreased kidney function and hypertension in adults (26, 27). Additionally, the prevalence of hypertension was correlated with circulating PCDD and PCDF concentrations in adults with dioxin exposure (28).

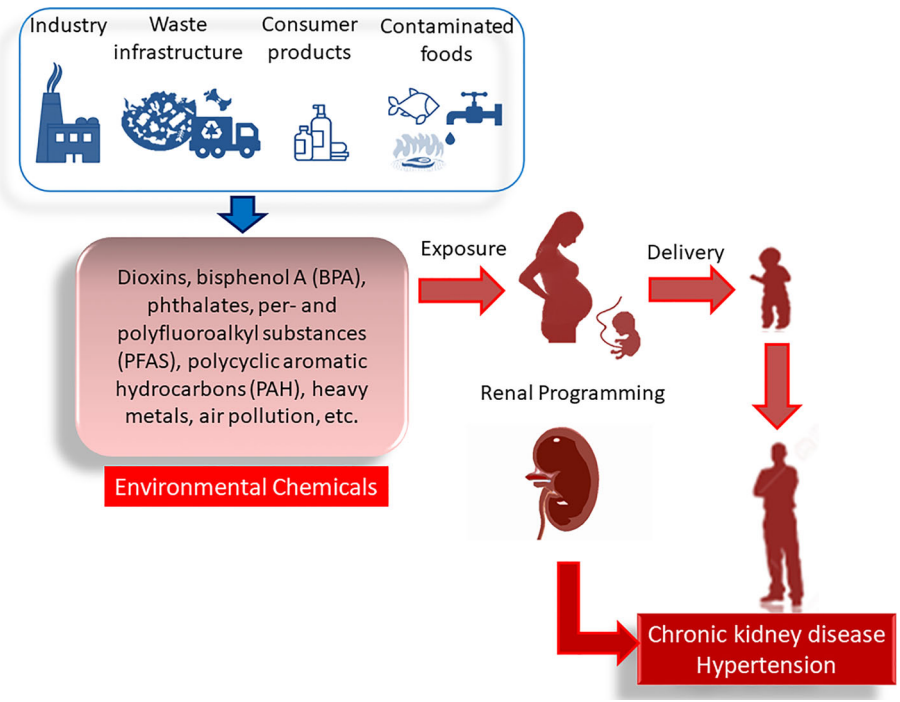

FIGURE 1 | Adverse impact of maternal environmental chemical exposure on developmental origins of kidney disease and hypertension. In pregnancy, exposure to various environmental chemicals occurs through daily consumer activity. There are many sources of contamination like industry, waste infrastructure, consumer products, contaminated foods, etc. These environmental chemicals cause renal programming, resulting in chronic kidney disease and hypertension in adulthood.

Nevertheless, the association between dioxins on kidney function and blood pressure (BP) in children remains largely unknown. The effects of dioxins are mainly mediated by the aryl hydrocarbon receptor (AHR)—a ligand-activated transcription factor that contribute to the pathogenesis of CKD and hypertension (59, 60).

2.2 Bisphenol A

Bisphenol A (BPA) was initially designed as a synthetic estrogen. It is now widely used for lining metal cans and in polycarbonate plastics, such as baby bottles, intravenous tubing, and dialysis circuits (29). Incomplete polymerization and polymer degradation of BPA causes it to leach out of food and beverage containers. BPA can be absorbed through ingestion, respiration, and the skin contact (30). As human exposure to BPA is frequent and widespread, more than 90% of individuals have detectable amounts of BPA in their urine (31). In humans, free BPA is rapidly metabolized in the liver and eliminated by renal excretion (32). High BPA concentrations have been reported in uremic patients received hemodialysis or peritoneal dialysis (32). Additionally, urinary BPA level was associated negatively with the estimated glomerular filtration rate (eGFR) and positively with BP (33, 34).

At concentrations lower than that reported in toxicological studies, BPA could provoke different endocrine-disrupting effects (30). BPA acts as an endogenous estrogen by interacting with estrogen receptors. Also, BPA is a ligand for the AHR. Thus, taking into account that endocrine disruption chemical (EDC) function as environmental signals and can be passed on to subsequent generations (61), there will be a growing need to understand the mechanisms of BPA action in order to decipher the association between maternal BPA exposure and kidney health in adult offspring.

2.3 Phthalates

Phthalates are a family of EDCs generally used as plasticizers in various industrial commodities (35). Low-molecular weight (LMW) phthalates have 3–6 carbon atoms in the backbone of their structure, whereas high-molecular weight (HMW) phthalates have 7–13 backbone carbons. LMH phthalates are frequently added to cosmetics, shampoos, and other personal hygiene products. HMW phthalates are commonly used to make vinyl plastics in applications in flooring, food packaging and intravenous tubing (35). Phthalates can be delivered to the human body through diet, inhalation, and skin contact. Di-2-ethylhexylphthalate (DEHP) and di-n-butyl phthalate (DBP) are the primary phthalate ester pollutants in the environment (36). The metabolites of phthalates can cross the placenta and be transferred to the fetus (37). Epidemiological studies demonstrated that high urinary DEHP levels are associated with high BP, low eGFR and albuminuria (38–40). As phthalates have estrogenic or antiandrogenic properties,

TABLE 1 | Major source and exposure-related adverse renal outcomes of environmental chemicals.

Environmental chemicals	Common substances or derivatives	Major source	Exposure-related adverse renal outcomes	References
Dioxins	TCDD, PCDD, PCDF, PCB	Consumption of animal products with high fat content, manufacturing of pesticides, bleaching of wood pulp and waste incineration	Reduced kidney function, albuminuria, hypertension	(24–28)
Bisphenol A		Plastic containers, lenses, medical tubing and devices	Reduced kidney function, albuminuria, hypertension	(29–34)
Phthalates	DEHP, DBP	Vinyl plastics, shampoos, cosmetics, food packaging, medical tubing and devices	Reduced kidney function, albuminuria, hypertension	(35–40)
Per- and polyfluoroalkyl substances	PFOA, PFOS	Electrochemical fluorination, telomerization, surfactants, food packaging, non-stick cooking surfaces, surface protection agents, fire-retarding foams	Reduced kidney function, hypertension	(41–43)
Polycyclic aromatic hydrocarbon	BaP	Cigarette smoke, incomplete combustion of coal, oil, and gas; charbroiled meat	Reduced kidney function, albuminuria, hypertension	(44–50)
Heavy metals	Pb, Cd, Hg	Lead: soil and dust (paint, gasoline, industrial sources); drinking water, cigarette smoke; Cadmium: fossil fuel combustion; phosphate fertilizers; batteries; contaminated food; Mercury: coal-fired power plants; smelters, municipal waste incineration	Reduced kidney function, albuminuria, hypertension	(17, 51–53)
Air pollution	PM_{10}, $PM_{2.5}$	Burning of fossil fuels, industrial processes, solvent use, agriculture, waste treatment	Reduced kidney function, hypertension	(54–58)

TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCDD, polychlorinated dibenzo-p-dioxin; PCDF, polychlorinated dibenzo-p-furan; DEHP, di-2-ethylhexylphthalate; DBP, di-n-butyl phthalate; BaP, benzo(a)pyrene; Pb, lead; Cd, cadmium; Hg, mercury; PM_{10} (particulate matter <10 μm in diameter), $PM_{2.5}$ (particulate matter <2.5 μm).

emerging evidence suggests the associations between prenatal phthalate exposure and adverse offspring outcomes (37). Following these findings, steps should be taken to explore the effect of phthalate exposure during pregnancy on offspring kidneys.

2.4 Per- and Polyfluoroalkyl Substances

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of human-made chemicals used in a broad range of consumer and industrial products (41). PFAS exposure is ubiquitous with perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) detectable in >90% of the population (42). For pregnant women, contaminated diet, drinking water, and air are the main sources of exposure. PFAS can be transferred from mother to fetus *in utero* and through breastfeeding to neonates (42). In adults, high PFOA or PFOS levels are associated with CKD (43). Likewise, elevated PFOA levels are associated with reduced kidney function in children and adolescents (62). Nevertheless, the association between blood PFOA and PFOS levels and hypertension was not identified in a pediatric cohort (63).

Several mechanisms have been linked to PFAS-induced kidney disease, including oxidative stress, peroxisome proliferators-activated receptor (PPAR) pathways, NF-E2–related factor 2 (NRF2) pathways, enhanced endothelial permeability, and epithelial mesenchymal transition (64). Of note, these mechanisms are also linked to developmental origins of kidney disease and hypertension (12–16). Despite emerging evidence portends PFAS are environmental threats to renal outcome; yet there is a gap in our understanding of whether maternal PFAS exposure affects offspring's kidney health.

2.5 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants and composed of two or more fused aromatic rings of carbon and hydrogen atoms, which come from industrial, mobile, domestic, and agricultural emission (44). PAHs are highly lipophilic and can easily accumulate in fat tissue of living organisms. Many PAHs are mutagenic, carcinogenic, teratogenic, and immunotoxic to humans (45). In pregnancy, comparable amounts of PAHs in maternal blood and cord blood, whereas low levels in placental tissue were found (44). These data indicates that PAHs can cross the placenta and transfer to the fetus. Another report illustrated that up to 30–95% of infants have exposure to PAHs by breastfeeding (45). Current evidence supports gestational exposure of PAHs is responsible for adverse birth outcomes like low birth weight and premature delivery (46). It has also been shown that benzo(a)pyrene (BaP) and other PAHs can increase stillbirths and congenital abnormalities (47). Regarding the kidney, studies in adults have identified increases in urinary PAH metabolites were associated with a decrease in eGFR (48), an elevation in BP (49), and the presence of albuminuria (50). Similar to many environmental chemicals, PAHs are known AHR ligands. Activation of PAH/AHR

signaling can alter the toxicokinetic profile of many nephrotoxic drugs, like aminoglycosides, to mediate kidney injury (65).

2.6 Heavy Metals

Heavy metals constitute an ill-defined group of inorganic chemical hazards, and those most commonly found at contaminated sites related to nephrotoxicity are lead (Pb), cadmium (Cd), and mercury (Hg) (17, 51). The general population is mainly exposed to lead from air and food, as lead in foodstuff originated from pots used for cooking and over 50% of lead emissions originating from petrol. Cadmium compounds are currently used as stabilizers and in re-chargeable nickel–cadmium batteries. Accordingly, cadmium exposure is generally from contaminated household waste and food; and cigarette smoking. Regarding mercury, the major source of exposure comes from contaminated food (i.e., fish) and dental amalgam. During pregnancy, there were greater accumulations of lead, cadmium, and mercury in the fetal kidney than in brain (52). Chronic exposure to lead has been linked to the development of lead nephropathy (53). Likewise, cadmium can cause nephrotoxicity *via* entering the renal epithelial cells (66). Mercury exposure has also been shown to elicit nephrotoxic effects like acute kidney injury and proximal tubule damage (17). In children, chronic relatively low-level exposure to various heavy metals may also increase the risk for CKD and hypertension (19, 20, 67). Owing to heavy metals remain the most important occupational and environmental pollutants, especially their nephrotoxic effects, there will be a growing need to understand whether maternal exposure to heavy metals impact renal outcomes in adult progeny.

2.7 Air Pollution

Epidemiological studies have obviously established that air pollution contributes to cardiovascular morbidity and mortality (54). Air pollutants include gaseous pollutants (e.g., carbon mono oxide, oxides of nitrogen, ozone and sulfur dioxide) and particulate matters (PMs). The coarse fraction contains the particles with a size ranging from PM_{10} (<10 μm in diameter), $PM_{2.5}$ (<2.5 μm) to ultrafine particle ($PM_{0.1}$). A meta-analysis study suggested that BP was positively related to $PM_{2.5}$ exposure with an elevation of 1.393 mmHg, 95% CI (0.874-1.912) and 0.895 mmHg, 95% CI (0.49-1.299) per 10 μg/m increase for systolic and diastolic BP, respectively (55). Additionally, there are several studies showing association with various PMs and CKD (56–58). Despite the association between maternal air pollution exposure and birth defects has been addressed (68, 69), how early exposure to particulate matters may increase the risk of adverse renal outcome in offspring is still largely unknown.

3 PRENATAL ENVIRONMENTAL CHEMICAL EXPOSURE ON RENAL PROGRAMMING

All of the above-mentioned epidemiological evidence linking environmental chemical pollutants to kidney diseases and hypertension are from studies established in direct but not maternal exposure. Certain chemicals can impair nephrogenesis, resulting in low nephron endowment and a spectrum of defects in the kidney and urinary tract (70). Accordingly, developmental nephrotoxic effects can be expected during environmental chemical exposure of pregnant women. Any of these anomalies coinciding with reduced nephron number may have long-term sequelae such as kidney disease and hypertension in later life (70, 71). Although infants can be an increased risk of nephrotoxicity to elemental (e.g., mercury) or organic contaminants (e.g., melamine) (19, 72, 73), studies focusing on association for postnatal environmental chemical exposures (a time after completion of nephrogenesis) and adverse renal outcomes were excluded. Here, we summarize clinical and experimental studies regarding environmental chemical exposure in pregnancy related to adverse renal outcomes and hypertension in offspring.

3.1 Epidemiological Evidence

As shown in **Table 2**, very few human observational studies addressed maternal environmental chemical exposure implicating in offspring's BP and renal outcome (74–84). All epidemiological evidence are mother-child cohort studies and none of them have been observed until adulthood. Prior prospective studies on the associations of maternal exposure to BPA and phthalates with childhood BP showed inconsistent results (74–78). Some studies did not show any association of fetal exposure to BPA with childhood BP, while others showed fetal exposure to BPA was associated with higher diastolic blood pressure (DBP) (74, 75). Another study showed higher second trimester maternal urine BPA levels were associated with higher systolic blood pressure (SBP) in boys at the mean age of 9.7 years (76). In the same cohort study of 1,064 mother-child pairs, maternal urine phthalate concentrations were not associated with BP in boys but were associated with lower BP in girls (76). A study of 500 children, found that participants born to mothers had high urinary phthalate metabolite concentrations was associated with low SBP and DBP at age 4 (77). Another study similarly showed that maternal urinary phthalate metabolite levels were negatively associated with SBP z-scores in girls (77). These studies investigating the associations of maternal phthalate exposure with childhood BP reported sex specific effects (76–78).

Table 2 illustrates that maternal heavy metal exposure, especially lead, is related to adverse renal effects on children. One study of 1,194 mother-infant pairs has evaluated the effect of prenatal exposure to heavy metals and trace elements on childhood BP (79). Hg and Pb were not associated with childhood SBP at 3 to 15 years of age. Although Cd was not associated with childhood systolic BP overall, the inverse association between manganese and childhood SBP was stronger at higher levels of Cd (79). Two studies investigated the associations between maternal lead levels and renal outcomes in offspring (80, 81). One study found there were no associations between maternal lead levels and childhood BP or eGFR at 8-12 years of age. However, they observed maternal lead level was negatively associated with kidney volume in children (80). Another study reported there was an inverse association

TABLE 2 | Effects of maternal environmental chemical exposure on blood pressure and renal outcomes in children.

Chemicals	Study/country	Participants	Major findings	References
Bisphenol A	EDC birth cohort/South Korea	645 children	Maternal urinary BPA concentration during midterm pregnancy was associated with children's DBP at age 4	(74)
Bisphenol A	European HELIX cohort	1,277 children	Increases in DBP were observed with maternal BPA concentrations	(75)
Bisphenol A	Generation R Study/Netherlands	1,064 mother-child pairs	Maternal second trimester urinary BPA levels were associated with SBP in boys at mean age 9.7 years	(76)
Phthalates	Generation R Study/Netherlands	1,064 mother-child pairs	Maternal urinary phthalate metabolite levels were negatively associated with SBP and DBP in girls	(76)
Phthalates	Rhea pregnancy cohort/Greece	500 mother-child pairs	Maternal urinary phthalate metabolite concentrations were negatively associated with SBP and DBP at age 4.	(77)
Phthalates	INMA birth cohort/Spain	391 mother-child pairs	Maternal urinary phthalate metabolite were associated with lower SBP z-scores in girls but not in boys.	(78)
Heavy metals	Boston Birth Cohort/USA	1,194 mother-infant pairs	Hg, Pb, and Cd were not associated with childhood SBP at 3 to 15 years of age.	(79)
Lead	MINIMat trial/Bangladesh	948 mother-infant pairs	There were no associations between maternal lead levels and childhood BP or eGFR at 8-12 years of age. There was an inverse association between maternal lead level and kidney volume.	(80)
Lead	PROGRESS birth cohort/Mexico	453 mother-child pairs	There was an inverse association between maternal blood lead levels and eGFR in overweight children at 8-12 years of age.	(81)
Air pollution	CANDLE study	822 mother-child pairs	The SBP percentile increased by 14.6 and DBP percentile increased by 8.7 with each 2-$\mu g/m^3$ increase in second-trimester $PM_{2.5}$.	(82)
Air pollution	PROGRESS birth cohort/Mexico	537 mother-child pairs	A 10 $\mu g/m^3$ increase in $PM_{2.5}$ predicts a cumulative increase of 2.6 mmHg in SBP and 0.88 mmHg in DBP at ages 4-6 years.	(83)
Air pollution	Boston Birth Cohort/USA	1,293 mother-child pairs	A 5 $\mu g/m^3$ increment in $PM_{2.5}$ during the third trimester was associated with a 3.49 percentile increase in childhood SBP at 3 to 9 ages of age.	(84)

EDC, Environment and Development of Children; INMA, Infancia y Medio Ambiente"—Environment and Childhood; HELIX, Human Early-Life Exposome; MINIMat, Maternal and Infant Nutrition Interventions, Matlab; PROGRESS, Programming Research in Obesity, Growth, Environment and Social Stressors; CANDLE, Conditions Affecting Neurocognitive Development and Learning in Early Childhood; SBP, systolic blood pressure; DBP, diastolic blood pressure.

between maternal blood lead levels and eGFR in overweight children at 8-12 years of age (81).

Regarding air pollution, one report demonstrated that higher prenatal $PM_{2.5}$ exposure, particularly in the second trimester, was associated with elevated childhood BP at 4-6 years of age (82). Another report similarly showed that second and third trimester $PM_{2.5}$ exposure may increase children's BP at 4-6 years of age (83). Analysis of one study of 1,293 mother-child pairs indicated that a 5-$\mu g/m^3$ increment in $PM_{2.5}$ during the third trimester was associated with a 3.49 percentile increase in childhood systolic BP at 3 to 9 ages of age (84) (**Table 2**).

So far, there is lack of information about the BP and renal outcomes in children born to mothers exposed to PFOA, PFNA, or PAHs. However, maternal exposure to these chemicals have been linked to preterm birth, low birth weight (LBW), and intrauterine growth retardation (IUGR) (85–87). It is noteworthy that these risk factors related to reduced nephron number (70, 71) as well as kidney disease and hypertension in later life (11, 72, 88, 89). Likewise, prenatal $PM_{2.5}/PM_{10}$ or phthalate exposure were related with IUGR and LBW (76, 90). Since developmental origins of kidney disease can be attributed to multiple hits, a programmed low nephron endowment likely constitutes a first-hit to the kidney which makes the remaining glomeruli more vulnerable to environmental influences and increases the risk for developing CKD when facing other chemical pollutants in later life.

3.2 Evidence from Animal Models

To establish a causal relationship between prenatal exposure to environment chemicals and kidney disease and hypertension, animal models are valuable tools for establishing the dose–response relationship, understanding the mechanisms of developmental programming, and developing therapeutic interventions (15).

Table 3 summarizes animal studies demonstrating the association between maternal environmental chemical exposure and subsequent kidney disease and hypertension in progeny (91–105). The current review is solely restricted to chemical exposures happening during the duration of kidney development, with a focus on reporting offspring outcomes starting after birth. As shown in **Table 3**, rats have been the dominant animal species used. However, using large animals to study similar exposures are not applied as of today. The programming effects of environmental chemicals have been reported in rats ranging from 2 to 21 weeks of age, which is roughly equivalent to human ages from infancy to young adulthood (106).

Several types of chemicals have been evaluated, including TCDD (88–90), BPA (94, 95), DEHP (96), DBP (97, 98), BaP (99), heavy metal mixture (100), Cd (101–103), and $PM_{2.5}$ (104, 105). Maternal exposure to TCDD or BPA causes the rise of BP in adult rat offspring (91, 92, 94), which was relevant to dysregulated AHR signaling pathway. Besides, hydronephrosis

TABLE 3 | Summary of animal models of developmental programming of kidney disease and hypertension categorized according to environmental chemical exposures.

Enviromental Chemical	Animal Models	Species/ Gender	Age at evaluation	Offspring Outcomes	Ref.
TCDD	TCDD 200 ng/kg orally on gestational days 14 and 21 and postnatal days 7 and 14	SD rats/M	12 weeks	Hypertension	(91)
TCDD	TCDD 200 ng/kg orally on gestational days 14 and 21 and postnatal days 7 and 14	SD rats/M	16 weeks	Hypertension	(92)
TCDD	TCDD 6.0 μg/g orally on gestational day 14.5	C57BL/6N mice/M	3 months	Hydronephrosis	(93)
BPA	Oral administration of bisphenol A 50 μg/kg/day during pregnancy and lactation.	SD rats/M	16 weeks	Hypertension	(94)
BPA	BPA 10 or 100 μg/kg/day during gestational days 9-16	OF1 mice/M & F	30 days	Impaired glomerular and tubular formation	(95)
DEHP	Oral administration of DEHP 0.25 or 6.25mg/kg/day during pregnancy	Wistar rats/ M & F	21 weeks	Reduced kidney function, reduced nehron number, and hypertension	(96)
DBP	Oral administration of DBP 850 mg/kg/day during gestational days 14–18.	SD rat/M	8 weeks	Reduced kidney function and renal fibrosis	(97, 98)
BaP	Oral administration of BaP 600 or 1200 μg/kg/day during gestational days 14-17	LEH rats/M & F	8 weeks	Hypertension	(99)
Heavy metals	Metal mixtures (Pb 125 or 250 mg/L, Cd 37.5 or 75 mg/L, Hg 0.75 or 1.5 mg/L) in drinking water during pregnacy and lactation	SD rats/M &F	23 days	Kidney injury and renal hypertrophy	(100)
Cd	Inhaled Cd oxide nanoparticle (230 μg CdO NP/m^3) for 2.5 h/d, 7 d/wk during gestational days 4.5-16.5	CD-1 mice/ M & F	14 days	Kidney injury	(101)
Cd	Oral administration of Cd chloride 0.5 mg/kg/day during pregnancy	Wistar rats/ M & F	60 days	Reduced kidney finction	(102)
Cd	Oral administration of Cd chloride 2.0 or 2.5 mg/kg/day on gestational days 8, 10, 12 and 14	SD rats/M	49 days	Kidney injury	(103)
$PM_{2.5}$	Oropharyngeal drip of $PM_{2.5}$ (1.0 mg/kg) at gestational days 8, 10, and 12	SD rats/M	14 weeks	Hypertension	(104)
$PM_{2.5}$	$PM_{2.5}$ exposure for 16 weeks before delivery	C57BL/6N mice/M & F	12 weeks	Hypertension	(105)

Studies tabulated according to types of environmental chemicals, animal models and age at evaluation. TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BPA, bisphenol A; DEHP, di-2-ethylhexylphthalate; DBP, di-n-butyl phthalate; BaP, benzo(a)pyrene; Pb, lead; Cd, cadmium; Hg, mercury; SD, PM_{10} (particulate matter <10 μm in diameter), $PM_{2.5}$ (particulate matter < 2.5 μm); Sprague-Dawley rat; LEH, Long Evans Hooded.

was described in rat offspring prenatally exposed to TCDD (93). EDC exposure during pregnancy induced kidney disease and hypertension in adult offspring was observed in three studies where BPA, DEHP, and DBP were orally administered in mother rats (95–98). Another environmental chemical that has been investigated is BaP (99). Oral doses of BaP exposure (600 or 1200 μg/kg/day) were administered to dams during gestational days 14-17 and showed hypertension in rat offspring of both sexes at 8 weeks of age (99). Animal studies of maternal heavy metal exposure implicating in the offspring kidney suggested that Cd is the main cause of adverse renal outcomes programmed by early-life heavy metal exposure (100–103). A combined metal mixtures (Pb, Cd, and Hg) in drinking water administered to mother rats during pregnancy and lactation study in rats resulted in kidney injury and renal hypertrophy in their offspring (100). Additionally, prenatally Cd-exposed offspring rats presented the features of kidney injury in other three studies (101–103), while no prior studies have addressed the effects of Pb or Hg. Furthermore, air pollution was shown to lead to hypertension in rats or mice prenatally exposed to $PM_{2.5}$ (104, 105).

3.3 Mechanisms behind Developmental Origins of Kidney Disease and Hypertension

Taking all these evidences in consideration, various environmental chemical exposures in pregnancy can increase the risk of kidney disease and hypertension later in life. Considering that diverse maternal chemical exposures induce similar offspring renal outcomes, there might be some common mechanisms behind renal programming. Up to the present, a number of mechanisms of renal programming have been identified and some of them are linked to the pathogenesis underlying environmental chemical-induced kidney disease and hypertension (12–15, 72, 107–110). Several mechanisms have been considered, including oxidative stress, aberrant activation of the renin-angiotensin system (RAS), reduced nephron numbers, and dysregulated AHR signaling pathway, as illustrated below (**Figure 2**). These mechanisms are discussed in the following sections.

3.3.1 Oxidative Stress

Oxidative stress is referred to overproduction of reactive oxygen and nitrogen species (ROS/RNS) prevails over the defensive antioxidant system, resulting in oxidative stress damage (111). ROS/RNS play a dual role in pregnancy; such as moderate ROS/RNS levels contribute to normal organogenesis, whereas their overproduction adversely affects fetal outcomes (112). There are several models of maternal chemical exposure tied up with oxidative stress in mediating kidney disease and hypertension of developmental origins, comprising TCDD (91, 92), BPA (94), and $PM_{2.5}$ (104). Increased ROS generation, decreased antioxidant capacity, and impaired nitric oxide (NO) signaling

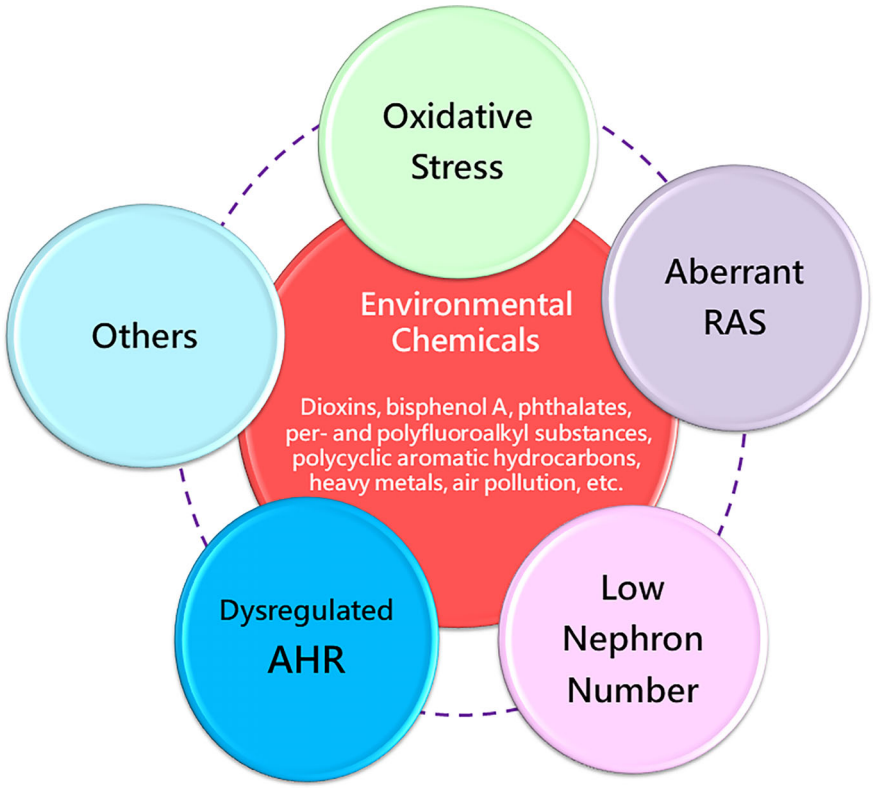

FIGURE 2 | Overview of the common mechanisms of renal programming in response to various environmental chemicals in early life. RAS, renin-angiotensin system; AHR, aryl hydrocarbon receptor.

pathway all contribute to oxidative stress-induced renal programming, as we reviewed elsewhere (108). A marker of oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OHdG), was increased in offspring kidneys prenatally exposed to TCDD (91) or BPA (94). Conversely, various antioxidants have been used as a therapeutic strategy to prevent developmental origins of kidney disease and hypertension (113). In a prenatal $PM_{2.5}$ exposure rat model (104), offspring developed hypertension coinciding with oxidative stress, which was prevented by tempol, a synthetic antioxidant. These findings support the notion that kidney disease and hypertension programmed by maternal chemical exposure might be attributed to oxidative stress. In view to oxidative stress is proposed as one of the main mechanisms of chemical-induced pathology in humans (114), its role in kidney disease and hypertension of developmental origins, especially in response to various prenatal chemical pollutants, awaits further exploration.

3.3.2 Reduced Nephron Number

Nephron number is a major determinant of kidney health in later life. In general, nephron number is approximately 1 million per human kidney, with a huge individual differences ranging from 0.2 to 2.5 million (115). As we mentioned earlier, prior research has demonstrated that reduced nephron number, in relation to LBW and preterm birth, may result in hypertension and kidney disease in later life (11, 72, 88, 89). Epidemiological studies demonstrated that maternal exposure to PFOA, PFNA, PAHs, phthalates, and $PM_{2.5}$/PM_{10} associated with preterm birth and LBW (76, 85–87, 90), both are risk factors related to reduced nephron number. Therefore, the role of these chemicals on nephron number in kidney disease and hypertension of developmental origins is still awaiting discovery but is certainly a subject of great interest.

Reduced nephron number can cause compensatory glomerular hyperfiltration and glomerular hypertension, consequently resulting in further nephron loss later in life. Accordingly, reduced nephron number has been found to be a key mechanism behind renal programming (72). In a maternal DEHP exposure model, adult offspring displayed reduced kidney function and hypertension coinciding with dysregulation of several nephrogenesis gene expression (96). These data suggest that maternal DEHP exposure impaired nephrogenesis, resulting in a nephron deficit, and subsequently kidney disease and hypertension later in life (96). Moreover, the severity of adverse nephrotoxic effects and the extent of renal involvement may be modified by the stage of kidney development (20). Thus, whether nephron number can be influenced by various chemical exposures in a dose- and stage-specific manner are required for further evaluation.

3.3.3 Aberrant Activation of RAS

The kidney is a major target for the multiple elements of the RAS (116). Blockers of the RAS have been the cornerstones of pharmacologic treatment for patients with hypertension and CKD (116). During nephrogenesis, constituents of the RAS are highly expressed and play key roles in mediating proper renal morphology and physiological function (117). As reviewed elsewhere (110), a transient biphasic response with downregulation of classical RAS axis in neonatal stage that becomes normalized with age. Thus, varied maternal insults can disturb this normalization in adulthood, insomuch that the classical RAS axis is inappropriately activated resulting in adult kidney disease and hypertension. While RAS blocker fetopathy, which presents renal malformation, appears when pregnant women taking angiotensin-converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB) during the nephrogenesis stage (118). **Table 3** shows several environmental chemicals can program the kidney and RAS concurrently—TCDD (92), DEHP (96), and BaP (99)—giving rise to hypertension in adult offspring. Currently, several early-life interventions targeting the RAS to prevent kidney disease and hypertension have been employed in animal models (110). To what extent the RAS are interconnected with various environmental chemicals towards kidney disease and hypertension of developmental origins are issues that await further clarification.

3.3.4 Dysregulated AHR Signaling Pathway

Quite a few environmental chemicals are ligands for AHR, such as TCDD, PCDD, PCDF, PCB, BPA, BaP (119). In addition to exogenous ligands (i.e., environmental chemicals), AHR signaling can be activated by endogenous ligands like tryptophan metabolites (119). In patients with kidney disease, the most important AHR ligands are uremic toxins, especially those gut microbiota-derived from tryptophan metabolism. These tryptophan-derived uremic toxins have proinflammatory, prooxidant, procoagulant, and pro-apoptotic effects, all of which are involved in the pathogenesis of hypertension and CKD (120). In a maternal BPA exposure model, adult offspring developed hypertension coinciding with increased AHR protein level as well as the mRNA expression of AHR target gene *Ahrr, Cyp1a1*, and *Arnt* (94). Similarly, maternal TCDD-induced programmed hypertension was associated with mediation of the AhR signaling pathway (91, 92). Conversely, antagonizing AHR signaling by resveratrol has been reported to protect adult offspring against hypertension programmed by environmental chemicals like TCDD (92) and BPA (94). Moreover, AHR signaling can modulate pro-inflammatory T helper 17 (TH17) axis and trigger inflammation, by which environmental chemicals may link to the development of hypertension and kidney disease (121, 122). Hopefully, elucidation of the role of AHR in chemical-induced programmed kidney disease and hypertension will aid in the development of novel therapies.

3.3.5 Others

Other molecular mechanisms relevant to the development of kidney disease and hypertension are identified in different animal models of developmental origins, such as dysbiotic gut microbiota (123), dysregulated nutrient-sensing signaling (124), impaired sodium transport (12), and epigenetic regulation (125). Since these mechanisms are more or less related to environmental chemicals (126–128), there might be considerable interplay among these mechanisms behind kidney disease and hypertension of developmental origins, even though this remains speculative.

4 THERAPEUTIC STRATEGIES TARGETING ON ENVIRONMENTAL CHEMICALS

Taking into account the fact that our advanced understanding of the DOHaD research recently, it turns out therapeutic interventions can be shifted from adulthood to early life before disease occurs, by so-called reprogramming (129). So far, reprogramming strategies to reverse the programming processes that have been investigated include lifestyle modification, nutritional intervention, and pharmacological therapy. Concerning environmental chemical pollutants, there is no doubt that reprogramming strategies should focus on avoiding exposure to theoretically harmful chemicals prenatally and promoting a healthy lifestyle. As mentioned earlier, several chemicals induced programmed kidney disease and hypertension are associated with oxidative stress (91, 92, 94, 104). Several natural antioxidants have been used as nutritional interventions in pregnancy to prevent kidney disease and hypertension in a number of animal models, as we reviewed elsewhere (113, 130). Additionally, early-life interventions targeting specific signaling pathways might be of benefit in the prevention of chemical pollutant-induced renal programming. An example of therapeutic target is the RAS. Several RAS-based interventions have also shown benefits in protecting against programmed hypertension, such as renin inhibitor, ACE inhibitor, ARB, and ACE2 activator (110). In view of that aberrant RAS signaling contributes to maternal chemical exposure-induced renal programming (91, 96, 99), RAS-based interventions might be an ideal reprogramming strategy. Furthermore, resveratrol acting like an AHR antagonist benefits kidney disease and hypertension of developmental origins (131, 132). Although various reprogramming interventions that show tremendous advances with regard to renal programming, their protective benefits against kidney disease and hypertension programmed by maternal environmental chemical exposure remain still a long way off.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Previous studies have indicated the adverse impact of environmental chemicals on public health. This review sought to highlight the risks of environmental chemicals are communicable to the future generations and the value of DOHaD approach will aid in prevention rather than treatment

of kidney disease and hypertension. At face value, it would be logical to consider early prohibiting exposure to hazardous chemicals. However, there are many aspects still unsolved. Limited environmental chemicals have been evaluated in humans and animal models of kidney disease and hypertension, not to mention that only a few of them have been studied in the DOHaD filed. Global chemicals production is expected to double by 2030, and the already widespread use of chemicals is likely to also increase, including in consumer products (133). At a deeper level, little reliable information currently exists regarding the long-term effects of environmental chemical exposure in human cohorts and animal studies. Most epidemiological evidence are mother-child cohorts, which are hard to proceed to adulthood. Considering certain chemicals like EDCs have shown transgenerational epigenetic effects on endocrine function, future work in animal studies is needed to better understand various environmental chemicals can induce kidney disease and hypertension in future generations to which extent. Moreover, reprogramming interventions targeting common mechanisms to prevent kidney disease and hypertension are still missing in the literature.

Peace, dignity and equality on a healthy planet — these are the ultimate goals stated by the United Nations in 2015, to be achieved by 2030 (134). While much remains to be done to tackle the challenging of NCDs, kidney disease in particular (135, 136). In 2020, the World Kidney Day informed the public about the importance of preventive interventions – be it primary, secondary or tertiary (137). Seeing the prevention strategy from a DOHaD perspective, primary and secondary prevention seems our best strategy to improve global kidney health. First, primary prevention aims to prevent kidney disease before it ever occurs. There is an urgent need for multidisciplinary efforts to perform investigations that identify toxic chemicals in the environment. During pregnancy through early childhood, avoiding harmful chemicals and toxins exposure at home, at work, and at play are essential for supporting kidney health. Although various environmental chemicals have been identified so far, preventive efforts should continue to discover other potentially harmful chemicals. Secondary prevention is early screening to identify and prompt treatment of kidney disease in the earliest stages. Although early detection CKD has the potential to yield marked public health benefits, most countries had inadequate CKD detection and surveillance systems to achieve this goal (136). Additionally, there will be a growing need to translate effective reprogramming interventions from animal studies into clinical practice as the process moves far slower than expected.

In conclusion, maternal environmental chemical exposure is a considerably pathogenetic link in kidney disease and hypertension of developmental origins. Further advances in the DOHaD field, aimed at the pregnant mothers and their offspring, hence have the potential to combat the burden of kidney disease and hypertension, which represent major global health challenges.

AUTHOR CONTRIBUTIONS

C-NH contributed to concept generation, data interpretation, methodology, drafting of the manuscript, critical revision of the manuscript, and approval of the article. Y-LT contributed to concept generation, methodology, drafting of the manuscript, critical revision of the manuscript, and approval of the article. All authors contributed to the article and approved the submitted version.

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Umbilical Cord Blood Leptin and IL-6 in the Presence of Maternal Diabetes or Chorioamnionitis

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Diabetes during pregnancy is associated with elevated maternal insulin, leptin and IL-6. Within the placenta, IL-6 can further stimulate leptin production. Despite structural similarities and shared roles in inflammation, leptin and IL-6 have contrasting effects on neurodevelopment, and the relative importance of maternal diabetes or chorioamnionitis on fetal hormone exposure has not been defined. We hypothesized that there would be a positive correlation between IL-6 and leptin with progressively increased levels in pregnancies complicated by maternal diabetes and chorioamnionitis. To test this hypothesis, cord blood samples were obtained from 104 term infants, including 47 exposed to maternal diabetes. Leptin, insulin, and IL-6 were quantified by multiplex assay. Factors independently associated with hormone levels were identified by univariate and multivariate linear regression. Unlike IL-6, leptin and insulin were significantly increased by maternal diabetes. Maternal BMI and birth weight were independent predictors of leptin and insulin with birth weight the strongest predictor of leptin. Clinically diagnosed chorioamnionitis and neonatal sepsis were associated with increased IL-6 but not leptin. Among appropriate for gestational age infants without sepsis, IL-6 and leptin were strongly correlated (R=0.6, P<0.001). In summary, maternal diabetes and birth weight are associated with leptin while chorioamnionitis is associated with IL-6. The constraint of the positive association between leptin and IL-6 to infants without sepsis suggests that the term infant and placenta may have a limited capacity to increase cord blood levels of the neuroprotective hormone leptin in the presence of increased cord blood levels of the potential neurotoxin IL-6.

Keywords: development, inflammation, insulin, neonatal, obesity, sepsis, adipokine cytokine

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INTRODUCTION

Cord blood analysis is used to identify potential links from intrauterine exposures to long-term outcomes. The cord blood levels of some hormones, such as insulin, primarily reflect production by the fetus (1), while other hormones, including the adipokines leptin and IL-6 (2), can enter the fetal circulation from the placenta (3, 4). Interpretation of cord blood levels may thus be confounded by maternal adiposity and alteration in placental function. For example, diabetes and inflammation increase placental production of leptin and IL-6 (5, 6), two hormones with structural similarities (7), but very different effects on neurodevelopment (8, 9).

Leptin is a pleiotropic hormone that is a key regulator of energy balance through the hypothalamic-pituitary-adrenal axis. As an adipokine, leptin is found in higher levels in obese individuals, including obese pregnant women (10–13). While maternal leptin can be transferred across the placenta, fetal leptin levels only weakly correlate with maternal levels (14–16), consistent with the presence of redundant sources to provide leptin to the fetus, including the placenta itself (17).

Cord blood leptin has been associated with reduced insulin sensitivity of the fetus (15), and when adjusted for the confounding effects of maternal BMI and birth weight, increased cord blood leptin levels have been inversely related to childhood lean mass index and directly correlated with adiposity, but the associations have attenuated with age (14, 18). In animal models, excessive leptin exposure early in life can lead to a state of persistent leptin resistance and obesity in adulthood while leptin deficiency can interfere with organ growth and neurodevelopmental outcomes (19, 20). In many respects, the growth promoting and signaling effects of leptin mirror those of insulin (21).

Beyond its well characterized metabolic and trophic effects, leptin has an increasingly recognized role in inflammation (22, 23). From a structural standpoint, leptin is similar to acute phase reactants, including the proinflammatory cytokine interleukin-6 (IL-6) (7), and leptin levels are rapidly increased by multiple cytokines (24, 25). Elevation in IL-6 levels is often found in cord blood of infants affected by maternal chorioamnionitis (26–28), suggesting leptin could be similarly impacted. In contrast to the stimulatory effects of IL-6 on leptin, physiologic-range hyperleptinemia has been shown to attenuate the IL-6 response to endotoxemia, suggesting cord blood IL-6 levels could be attenuated in large for gestational age infants that are characteristically hyperleptinemic (29).

Beyond acute inflammatory cascades, IL-6 has recently gained attention as a potential biomarker of the chronic inflammation that contributes to the development of diabetes or obesity (30, 31), but the potential for maternal diabetes to impact perinatal development through exposure to IL-6 has not been defined. Concerning preclinical findings that include increased placental IL-6 production followed by the development of neuropathology in a maternal immune activation model and offspring behavioral alterations and increased adiposity following isolated maternal IL-6 administration alone emphasize the importance of assessing not just leptin but also IL-6 levels across a variety of perinatal conditions (8, 32, 33). Because the two adipokines can have both augmentative and counterbalancing effects on different types of inflammation, for example leptin induces Th1 responses while IL-6 inhibits them, there is a critical need to obtain correlative clinical data (34–36).

The goal of this study was to evaluate the differential impact of growth, maternal diabetes, and chorioamnionitis on umbilical cord blood leptin and IL-6 levels. We utilized a high risk pregnancy cohort enriched with perinatal morbidities of interest to test the hypothesis that there is a positive correlation between cord blood IL-6 and leptin with progressively increased levels in pregnancies complicated by maternal diabetes and chorioamnionitis.

MATERIALS AND METHODS

Patient Selection

Using the University of Iowa IRB-approved Maternal-Fetal Tissue Bank database (IRB#200910784) (37), term infants were identified that met the inclusion criteria: birth following induction of labor and/or birth to a mother with diabetes during pregnancy. Plasma samples and corresponding clinical information were obtained for 104 infants (68 born following induction of labor and 36 born to women with diabetes). This study design was chosen to enrich the proportion of pre-specified morbidities, including large for gestational age birth weights, chorioamnionitis and maternal diabetes. Exclusion criteria included multiple gestation, fetal anomalies, maternal age less than 18 years, or inability to provide informed consent. The sample size was chosen to provide adequate power to detect moderate effect sizes (r=0.25) by simple linear regression and large effect sizes (f=0.4) by multiple regression models including up to 8 predictors of cord blood leptin (maternal obesity, maternal diabetes, maternal BMI, maternal weight gain, chorioamnionitis or sepsis, birth weight, cord blood insulin, and cord blood IL-6).

Clinical Data and Sample Collection

Maternal and infant data were collected from the electronic medical record (Epic, Verona, WI, USA). Maternal data included age at delivery, first trimester weight (at first presentation to obstetric care), weight gain during pregnancy, maternal age, and first trimester body mass index (BMI), later categorized as normal (18.5-24.9), overweight (25.0-29.9), or obese (>30). Data were also collected on the presence of chorioamnionitis or diabetes, including gestational onset diabetes mellitus treated with diet or medication. Infant data included sex, birth weight, gestational age, and newborn antibiotic use. Umbilical cord blood samples were collected at the time of delivery into cord blood collection bags from Fenwal Scientific (Fenwal Inc., Lake Zurich, Illinois, USA). Plasma was separated, snap frozen, and stored at -80 degrees Celsius until further analysis.

Plasma Analysis

Total protein content was quantified using the Pierce BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Leptin, insulin and IL-6 were quantified in duplicate using a custom multiplex panel (EMD Millipore, St Charles, MO, USA) on a BioRad analysis system (Bio-Rad Laboratories, Inc, Hercules, CA, USA) according to manufacturer's instructions. The assay detects minimum concentrations of 41 pg/ml for leptin, 87 pg/ml for insulin, and 11 pg/ml for IL-6. Cord blood levels were normalized to total protein content to correct for dilution that occurs during the harvest of cord blood into the anticoagulated collection system.

Statistical Analysis

All statistical analyses were performed with SigmaPlot 14.0 software (Systat Software, Inc, California) and confirmed with SPSS (Version 25.0, IBM Corp., Cary, NC). Data are expressed as mean +/- standard deviation. One-way analysis of variance and univariate linear regression were used to determine significant associations between hormone levels and categorical or continuous variables, respectively. A P value of <0.05 was considered statistically significant. When significant associations were present on univariate analysis, multiple regression was performed to identify independent predictors of hormone levels.

RESULTS

Demographic data and cord blood protein levels are summarized in **Table 1**. In this cohort, mean maternal BMI in the first trimester (30.4 kg/m^2) exceeded the 30 kg/m^2) cutoff typically used to define obesity. Mean maternal weight gain (12 kg) exceeded the recommendation for women with an initial BMI above 25 kg/m^2), and 45% of women had excessive weight gain during pregnancy. Mean birth weight was 3524 grams, and when corrected for gestational age and sex, this represented the 57th percentile.

By linear regression, cord blood leptin significantly correlated with cord blood insulin (R=0.50, P<0.001). There was no correlation between IL-6 and either leptin (R=0.08, P=0.40) or insulin (R=-0.02, P=0.86). Maternal BMI significantly correlated with leptin (R=0.30, P<0.01) and insulin (R=0.44, P<0.001), but not IL-6 (R=-0.04, P=0.67). Maternal weight gain was not associated with leptin (R=0.07, P=0.45), insulin (R=0.04, P=0.67) or IL-6 (R=-0.07, P=0.47). Birth weight significantly correlated with leptin (R=0.37, P<0.001) and insulin (R=0.27, P<0.01), but not IL-6 (R=-0.18, P=0.07).

TABLE 1 | Patient characteristics and hormone levels in cord blood samples.

	Mean ± SD
Maternal age, years	31.6 ± 5.5
Maternal BMI, kg/m2	30.4 ± 9.8
Maternal weight gain, kg	12.2 ± 6.9
Gestational age, weeks	39.5 ± 1.1
Infant's birth weight, grams	3524 ± 522
Total protein (TP), mg/ml	28 ± 10
Leptin, pg/ml	10499 ± 10486
Leptin/TP, pg/mg	436 ± 514
Insulin, pg/ml	391 ± 509
Insulin/TP, pg/mg	15 ± 19
IL-6, pg/ml	25 ± 45
IL-6/TP, pg/mg	0.91 ± 1.24

The prevalence of the perinatal outcomes of interest, and their associations with cord blood proteins are provided in **Table 2**. Maternal obesity, which was present in 39% of the cohort, was associated with a more than 2-fold increase in cord blood insulin. Likewise, maternal diabetes, which was present in 45% of the cohort, was associated with significant increases in cord blood leptin and insulin, but not IL-6. In contrast, clinically diagnosed maternal chorioamnionitis was associated with a 2-fold increase in IL-6. Antibiotics were prescribed for all 12 infants born to women diagnosed with chorioamnionitis and another 13 infants with suspected sepsis. The infants that received antibiotics had 2-fold increased IL-6 in their cord blood. There was no association between maternal chorioamnionitis or neonatal antibiotic use with cord blood leptin or insulin.

Of the pre-specified risk factors, only maternal chorioamnionitis and neonatal antibiotic use were associated with IL-6 levels. In contrast, multiple factors were associated with leptin and insulin, including maternal diabetes, maternal BMI and infant's birth weight. By multivariate regression (**Table 3**), birth weight was the strongest predictor of cord blood leptin (P<0.001), while maternal diabetes and BMI were the strongest predictors of cord blood insulin (both P<0.001).

To further assess IL-6 levels in the absence of the confounding effects of acute perinatal infection, additional analyses were performed after excluding the 25 infants that received antibiotics for suspected sepsis. By linear regression, cord blood IL-6 in those without suspected infection was significantly correlated with cord blood leptin (N=79, R=0.39, P<0.001) but not insulin (R=0.10, P=0.37). There was still no association between IL-6 and birth weight (R=0.01, P=0.96), but the association between leptin and IL-6 was strengthened when the analysis was restricted to appropriate for gestational age infants without sepsis (N=67, R=0.56, P<0.001, **Figure 1**) or infants without sepsis with birth weights at the 25th to 75th percentile (N=34, R=0.71, P<0.001). In the absence of sepsis, there was no association between IL-6 and maternal diabetes (P=0.49) or obesity (P=0.77).

DISCUSSION

The purpose of this study was to evaluate the association of two risk factors for impaired neonatal development, maternal diabetes and chorioamnionitis, with umbilical cord blood levels of two hormones that may contribute to long-term neurodevelopmental outcomes, leptin and IL-6. The results demonstrate that birth weight is the strongest predictor of cord blood leptin, with maternal BMI and diabetes having a greater influence on insulin than leptin. Cord blood leptin was unaltered

TABLE 2 | Perinatal risk factors and their association with cord blood protein levels, reported as pg/mg total protein.

	N (%)	Leptin, pg/mg	Insulin, pg/mg	IL-6, pg/mg
Maternal BMI				
Normal	38 (37)	305 ± 230	10 ± 10	0.8 ± 0.9
Overweight	25 (24)	475 ± 450	12 ± 16	1.1 ± 1.5
Obese	41 (39)	535 ± 695	22 ± 24*	0.9 ± 1.3
Maternal Diabetes				
No	57 (55)	302 ± 289	7 ± 7	1.0 ± 1.2
Yes	47 (45)	599 ± 664*	26 ± 23*	0.7 ± 1.2
Chorioamnionitis				
No	92 (88)	436 ± 524	16 ± 20	0.8 ± 1.0
Yes	12 (12)	439 ± 452	11 ± 10	1.7 ± 2.4*
Newborn Antibiotics				
No	79 (76)	468 ± 557	15 ± 18	0.7 ± 0.6
Yes	25 (24)	338 ± 336	17 ± 22	1.4 ± 2.2*

*$P<0.05$.

in the presence of clinically diagnosed infection but correlated with IL-6 in the absence of infection. In contrast, IL-6 was predominately associated with maternal chorioamnionitis, rather than birth weight or diabetes. This is the first study we are aware of that demonstrates an association between cord blood IL-6 and leptin in appropriate for gestational age infants without sepsis.

Birth weight remains the most widely available predictor of the long-term complications of potentially adverse prenatal exposures (38). In the presence of maternal obesity and diabetes, increased birth weight could be a consequence of the trophic effects of hormones, including insulin and leptin, as well as a manifestation of either genetic predisposition or enhanced nutrient availability. As expected, we noted the highest insulin levels in infants of diabetic mothers. This is typically ascribed to increased insulin production by the fetal pancreas in response to maternal hyperglycemia, although transplacental insulin passage may contribute (1, 39). As noted by others, we observed an independent association between maternal BMI and cord blood insulin which may reflect a role for subclinical insulin resistance in overweight or obese individuals that have not been diagnosed with diabetes (39–41).

As an adipokine that is also produced by the placenta, leptin's relationship with maternal and newborn anthropometrics is inherently more complex than that of insulin. Although we did not directly assess adiposity or placental morphology, birth weight and maternal BMI were both associated with cord blood leptin levels. Because our study was focused on term infants that would be expected to have expanding adipose depots, endogenous leptin production would be expected to significantly contribute to circulating leptin levels (42), and on multivariate analysis, birth weight was the strongest predictor of cord blood leptin. Our results are consist with the observations of Gross and colleagues as well as Persson and colleagues that demonstrated higher cord blood leptin in infants of diabetic mothers with a strong association with birth weight; those investigations also each replicated the results of others in showing no association between maternal leptin and cord blood leptin, again suggesting an important role for birth weight-related alterations in leptin production or metabolism (43–45). The correlation we observed between cord blood insulin and leptin is consistent with known role of insulin in the regulation of adipocyte leptin production and the previous reports of others (46, 47).

We compared leptin and IL-6 level across two common perinatal conditions that are coupled with inflammation, diabetes and chorioamnionitis. The association of leptin with diabetes but not chorioamnionitis is consistent with a more robust role for leptin in endocrine than infectious conditions, and a relatively diminished role for leptin in perinatal inflammatory cascades. That contrasts with the significant elevation in IL-6 that we found associated with chorioamnionitis or neonatal sepsis but not diabetes; this finding of increased IL-6 with presumed infection increases confidence that if leptin played a significant augmentative role in perinatal infection-related inflammation within our cohort, we would have noted altered cord blood levels or a correlation between IL-6 and leptin. Like leptin, IL-6 is an adipokine, but in contrast to leptin, IL-6 production by adipocytes typically requires an active inflammatory response that may be blunted

TABLE 3 | Generalized linear model with leptin or insulin as the dependent variable, maternal diabetes as a factor and maternal BMI or birth weight as potential covariates.

	Leptin		Insulin	
	Wald Chi-Squared	P	Wald Chi-Squared	P
Maternal Diabetes	5.1	0.02	27	<0.001
Maternal BMI	4.7	0.03	14.5	<0.001
Birth weight	12.5	<0.001	4.4	0.04

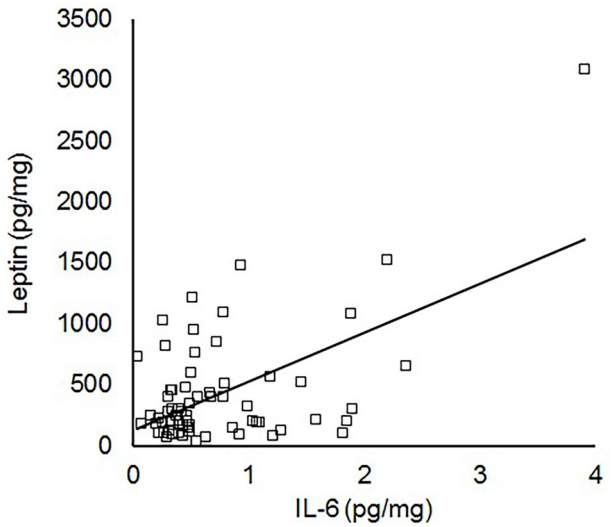

FIGURE 1 | Linear correlation between IL-6 and leptin within the cord blood of appropriate for gestational age term infants without sepsis (N=67, R=0.56, P<0.001).

at later stages of pregnancy (48, 49). Gestational diabetes-related immune dysregulation has been shown to lead to increased maternal IL-6 with enhanced placental leptin production but a paradoxical reduction in the IL-6 levels of the LGA offspring (30), possibly related to the hyperleptinemia that is typically seen in LGA infants (29).

This is the first investigation we are aware of that assessed the levels of leptin and IL-6, two adipokines with overlapping signaling pathways but divergent implications for neurodevelopment, in the context of common pregnancy complications. Leptin, a neurotrophic hormone, was increased primarily in association with birth weight and secondarily by maternal obesity and diabetes. IL-6, a potential neurotoxin, was increased in the presence of presumed perinatal infection but not maternal obesity or diabetes. These results clarify the unique predispositions towards adult disease that can follow major maternal morbidities and suggest leptin may not be biologically positioned to counterbalance the effects of IL-6 in the presence of chorioamnionitis. It is possible that our use of a high-risk cohort with a high rate of maternal diabetes and obesity may limit generalization to cohorts with a lower incidence of maternal morbidities. Future studies are also needed to assess the long-term implications of these and other hormones and to assess them at earlier gestational ages, when relatively lower fetal leptin production may unmask potential maternal obesity or diabetes-related alterations in IL-6 exposure during critical neurodevelopmental windows.

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 the University of Iowa's Institutional Review Board. The patients/participants provided their written informed consent to participate in this study

AUTHOR CONTRIBUTIONS

LV, BS, DS, MS, DB, DD, and RR contributed to conception and design of the study. LV, BS, DS, and DB organized the database. LV, BS, and RR performed the statistical analysis. LV and BS wrote the first draft of the manuscript. DS and RR wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Pre-Weaning Exposure to Maternal High-Fat Diet Is a Critical Developmental Window for Programming the Metabolic System of Offspring in Mice

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Background: Maternal high-fat diet (HFD) during pregnancy and lactation exerts long-term effects on the health of offspring. However, the critical developmental window for metabolic programming of maternal exposure to HFD on pathogenesis of obesity in offspring needs further clarification.

Materials & Methods: Female ICR mice were fed low-fat diet (LFD) or HFD for 8 weeks until delivery. During lactation, half of LFD dams received HFD while the other half of LFD dams and HFD dams maintained the previous diet. Male offspring were weaned at postnatal day 21 (P21) and fed LFD or HFD for 7 weeks. Metabolic parameters, biochemical, and histological indicators of thermogenesis, rectal temperature, and sympathetic nerve tone were detected at P21 and 10 weeks old.

Results: At P21, LH (maternal LFD before delivery but HFD during lactation) and HH (maternal HFD before delivery and during lactation) offspring gained more body weight and showed higher serum glucose and triglyceride levels as compared with LL (maternal LFD before delivery and during lactation), and the metabolic characters were maintained until 10 weeks age when fed with LFD after weaning. However, LH offspring exhibited a greater degree of metabolic abnormalities compared to HH offspring, with increased body weight, as well as lower norepinephrine (NE)-stimulated rectal temperature rise when fed with HFD after weaning. The lower UCP1 levels and HSL phosphorylation in LH offspring further suggested that brown adipose tissue (BAT) thermogenic function was impaired.

Conclusion: Exposure to maternal HFD feeding during pre-weaning period alone showed similar detrimental effects on programming metabolic system of offspring as those of both prenatal and early postnatal HFD feeding. Early postnatal stage is a critical

time window for metabolic programming and has profound and long-lasting effects on BAT development and function through sympathetic nerve-mediated thermogenesis.

Keywords: high-fat diet (HFD), gestation, lactation, offspring, thermogenesis

INTRODUCTION

Obesity has been considered as a worldwide health problem, being a major factor for several chronic diseases, namely, type 2 diabetes mellitus (T2DM), hypertension, and nonalcoholic fatty liver disease (1). Although genetic susceptibility, unhealthy lifestyle, high-calorie food intake, and exposure to adverse environments contribute to the development of obesity, an inter-, or trans-generational inheritance of abnormal metabolic state of parents may partially account for the current obesity epidemiology (2). For instance, studies have shown that maternal obesity induced by chronic HFD feeding before and throughout gestation and lactation have epigenetic programing effects, leading to metabolic dysfunction in offspring (3).

In mammals, the early postnatal period (from time of birth to weaning, also called pre-weaning period, corresponding to maternal lactation) exerts significant effects on maturation of multiple key organs (e.g., hypothalamus, adipose tissue, liver, and gut) (4–7). Additionally, adipose tissue dysfunction is a key factor in the metabolic alterations under the condition of obesity-caused early life insults (8). There are two main types of adipose tissues: white and brown adipose tissues (WAT and BAT) (9–11). The function of WAT is to store excess energy as triglycerides (TG). BAT is a major thermogenic center *via* dissipating energy in the form of heat to participate in the regulation of body weight. Brown adipocytes from BAT, along with their functionally-analogous beige or brite adipocytes have become the critical target against obesity and diabetes (12). Cold or food-intake stimulates sympathetic nerve system (SNS) to release noradrenaline (NE), which activates β3-adrenergic receptors (Adrb3) and increases the transcription of uncoupling protein 1 (UCP1) and thermogenic genes in brown adipocytes (13). In WAT, adipocyte lipolysis is activated by β3-adrenergic signaling through hormone-sensitive lipase (HSL) and release fatty acids participating in β-oxidation in BAT (14).

The progenitors of brown adipocytes originated from the central dermomyotome during early stages of embryogenesis. Brown fat pads were detectable in mid-gestation and then increased in size until birth. At postpartum, proliferating progenitors and adipocytes were further differentiated into mature brown adipocytes (15). Thus, the prenatal and early postnatal periods are considered important for development and maturation on BAT (16, 17). Previous studies have shown that both prenatal and early postnatal exposure to maternal HFD impaired the adaptive thermogenesis of BAT in offspring (18, 19). But the critical time window of maternal HFD exposure, prenatal, early postnatal, or both, contributed to metabolic phenotypes of offspring remains unclear, especially effects on BAT thermogenic function. Moreover, a recent study indicated that exposure to maternal HFD during pregnancy and lactation had markedly different effects on taste preferences of offspring (20). Other studies have shown that maternal HFD programs a NAFLD phenotype, which is critically dependent on the neonatal period (21). Therefore, the further study to clarify which stage exposure to maternal HFD plays a critical role in BAT of offspring is in demand.

Thus, we aimed to establish an animal model of metabolic programming to identify the critical window of BAT in response to maternal HFD feeding. Female mice received LFD or HFD during stages of gestation and lactation. Offspring are exposed to maternal LFD or HFD pre-weaning and then challenged with HFD post-weaning. Here, in this study, three maternal dietary intervention periods were involved: before delivery, lactation, and post-weaning. By this approach, changes in metabolic phenotype of offspring were detected at P21 and in adulthood. Specifically, we examined BAT thermogenic function of offspring when exposure to maternal HFD both prenatal and early postnatal or early postnatal alone. Our results show decreased expression of genes regulating thermogenesis and SNS signaling at both mRNA and protein levels in offspring when exposed to maternal HFD pre-weaning alone.

MATERIALS AND METHODS

Animals and Intervention

The animal studies were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals. All experimental procedures were approved by the Animal Care and Management Committee of the First Affiliated Hospital of Chongqing Medical University (Approval No- 2021-02). Five-week-old ICR female mice were randomly assigned to two groups. One group received LFD (10% energy from fat, 70% energy from carbohydrate, 20% energy from protein, D12450B, Research Diets, Inc.) and another group received HFD (45% energy from fat, 70% energy from carbohydrate, 20% energy from protein, D12451, Research Diets, Inc.) for 5 weeks before mating, with body weight (BW) measured weekly and water supplied *ad libitum*. The subsequent studies were conducted according to the schedule depicted in **Figure 1**. Female mice were subsequently bred with control male mice. Pregnant mice were maintained on their respective diet during gestation. On the day of delivery, pups (namely, both male and female pups) were adjusted to 10 per litter to assure the same nutrition availability for each litter, as small litter size increases the propensity for metabolic diseases in adulthood (22). Given the sex difference in the development of metabolic abnormality (23), only male offspring were involved in the next study. During lactation, a half of LFD dams received HFD feeding, the other half LFD dams and HFD dams maintained on

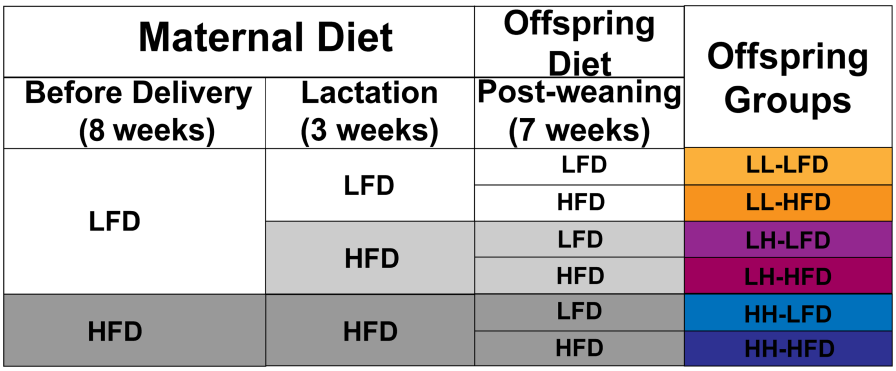

FIGURE 1 | The animal experimental schedule. Before mating, five-week-old ICR female mice were fed control LFD (10% kcal fat) or HFD (45% kcal fat with sucrose) for 5 weeks. The dams received the same diets during gestation. Dams fed gestation LFD received LFD or HFD, and dams fed gestation HFD received HFD during the lactation. The litters were adjusted to 10 pups per litter on the day of delivery. At weaning, three groups were produced: LL, offspring from both gestation and lactation LFD-fed dams; LH, offspring from gestation LFD-fed and lactation HFD-fed dams; or HH, offspring from both gestation ad lactation HFD-fed dams. And then, the male offspring from each group were selected to receive post-weaning LFD or HFD until they were 10 weeks old.

respective diet. Therefore, there were three groups involved before weaning: LL, HH, and LH. Pups from each group were weighed daily (P4–21) and were weaned at P21. Next, all dams and 2–3 pups from each group were euthanized at P21. The blood samples were taken to collect serum. The adipose tissues (interscapular BAT and inguinal WAT) were removed, weighed, and stored at −80°C. After weaning, the remaining pups were received LFD or HFD for 7 weeks until 10 weeks of age. We increased the number of mice receiving HFD because there might exist diet-induced obesity resistance (DIR) (24). Therefore, the study involved six groups: offspring from LL dams fed post-weaning LFD (LL-LFD, n = 10) or HFD (LL-HFD, n = 15), offspring from LH dams fed post-weaning LFD (LH-LFD, n = 10) or HFD (LH-HFD, n = 15), offspring from HH dams fed post-weaning LFD (HH-LFD, n = 10) or HFD (HH–HFD, n = 15). After 7 weeks of diet intervention, the offspring were divided into two groups. One group was intraperitoneally injected with norepinephrine (NE) (0.2 mg/kg) and the control group was injected with the same dose of glucose (GS). Then the rectal temperature was monitored before euthanized.

Glucose Tolerance Test (GTT)

Maternal mice and 10-week-old offspring were weighed and fasted for 10 h before testing. The baseline blood glucose levels were measured prior to intraperitoneal injection with glucose (2.0 mg/g Body Weight), and then blood glucose levels were measured at 0, 15, 30, 60, and 120 min post-injection with a glucometer (Roche Diagnostics).

Hematoxylin & Eosin (H&E) and Immunohistochemical Staining

The iWAT of offspring at P21 and both iWAT and BAT of the 10-week-old offspring were collected for standard fixation, paraffin-embedded, and vertical sectioned 4-μm thick for hematoxylin and eosin (H&E) staining or used for UCP1 immunohistochemical analysis in accordance with the standard protocol as previously described (25). Images were obtained with Olympus VS200 and the images were captured at ×200 magnification. For immunohistochemical staining, the UCP1-positive areas were determined by analyzing the staining intensity with ImageJ 1.5.1j8 and data were presented as a percentage of positive area.

Serum Biochemistry Determination

Blood samples, obtained from mice at weaning, were allowed to clot on ice and were centrifuged for 20 min at 2,000*g*, and serum was stored at −80°C until used. Serum triglycerides levels were determined with triglyceride colorimetric assay kit (Jiangsu enzyme label Biotechnology Co., Ltd, Jiangsu, China) according to the manufacturer's instructions.

Western Blotting

Proteins were extracted from BAT or iWAT using RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail (MCE) and phosphatase inhibitor cocktail (MCE). The protein concentrations were quantified using bicinchoninic acid assay (Beyotime). Loading protein samples were prepared using loading buffer and dithiothreitol. Proteins were run on SDS–polyacrylamide gel electrophoresis and then transferred to PVDF membranes. The membranes were blocked for 60 min and incubated overnight at 4°Cwith the appropriate primary antibodies. Subsequently, immuno-reactive proteins were blotted with anti-rabbit or anti-mice horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. The membranes were incubated with enhanced chemiluminescence reagent for 2 min (Thermo Scientific), visualized using Fusion FX Spectra system (Vilber Lourmat). Data analyzed using the ImageJ program, and relative quantification was normalized to β-ACTIN. Antibodies used are listed in **Table 1**.

RNA Extraction and Quantitative Real-Time PCR Analysis

RNA was extracted from BAT using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA integrity and quality were determined with agarose gel electrophoresis and a NanoDrop

TABLE 1 | Antibodies used for immunohistochemistry and western blotting.

Usage	Antibody	Species	Catalog no.	Dilution	Manufacturer
Primary antibody	anti-Ucp1	Mouse	sc-518024	1:1,000	SANTA CRUZ
	anti-p-HSL	Rabbit	4139	1:1,000	Cell Signaling Technology
	anti-HSL	Rabbit	4107	1:1,000	Cell Signaling Technology
	anti-β-ACTIN	Mouse	60008-2-Ig	1:10,000	Proteintech
Secondary antibody	anti-mouse IgG	Horse	7076	1:10,000	Cell Signaling Technology
	anti-rabbit IgG	Goat	7074	1:10,000	Cell Signaling Technology

2000 spectrophotometer (Thermo Scientific). cDNA was reverse-transcribed from 1 μg RNA using a reverse transcription kit (TaKaRa) in accordance with the manufacturer's instructions. For quantitative real-time PCR analysis (RT-qPCR), quantitative expression assays for genes were measured with SYBR Green (TaKaRa) using a Bio-Rad Real-Time System. The relative quantification was normalized and calculated with the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$). Primer sequences are listed in **Table 2**.

Rectal Temperature Measurement

To monitor the rectal temperature, an animal temperature controller with a rectal probe for mice was used. The mice were placed in 37°C constant temperature environment, and ketamine (55 mg/kg) plus xylazine(15 mg/kg) was injected intraperitoneally. After anesthesia, the probe was inserted about 5 cm into the anus of mice, and the temperature of mice was maintained at about 37°C for 5 min. Rectal temperature was measured every minute for 20 min after intraperitoneally injected with NE (0.2 mg/kg) or GS.

Statistical Analysis

Data are presented as the mean ± SEM. The statistical analyses were performed with GraphPad Prism (version 8.0). The significance of differences between groups were analyzed statistically using a one-way or two-way analysis of variance (ANOVA), followed by a Tukey's multiple-comparison *post hoc* test. For all analysis, $P < 0.05$ was considered statistically significant.

RESULTS

Exposure to Maternal HFD Both Prenatal and Early Postnatal or Early Postnatal Alone Predisposes the Offspring for Metabolic Disorders at P21

In our previous study, we measured maternal phenotype and metabolic parameters after HFD feeding on female ICR mice. As the results have shown, HFD dams gained more body weight but there was no significant difference in blood glucose, indicating HFD caused maternal obesity but did not impair glucose metabolism (26).

To confirm the relationship between maternal HFD and metabolism of the descendants, dams received LFD or HFD intervention during lactation, and then we measured the phenotype of pups at P21. The results suggest that pups from LH and HH dams gained more body weight at P9 compared with pups from LL dams, and such difference maintained throughout pre-weaning period (**Figure 2A**). At P21, pups from LH and HH dams had higher blood glucose level and higher blood triglyceride level than pups from LL dams, but there was no significant difference between pups from LH and HH (**Figures 2B, C**). Similarly, pups from LH and HH presented greater iWAT mass and higher ratio of fat mass to body weight (**Figures 2D, E**). Although pups from LH and HH had greater BAT mass than pups from LL, they had significantly lower ratio of BAT weight to body weight (**Figures 2D, E**). Furthermore, the histological analysis showed there were larger lipid droplets in iWAT of pups born to LH and HH dams (**Figure 2F**). Taken together, with exposure to maternal HFD both prenatal and early postnatal or early postnatal alone had transformed the morphology of adipose tissue and elevated the blood glucose level of pups at P21, which made them seemingly prone to developing obesity.

Offspring of LH Were Most Prone to Developing Obesity and Metabolic Disorder When Challenged With HFD Post-Weaning

To investigate long-term effects of pre-weaning HFD on offspring, offspring received HFD or LFD interventions for 7 weeks post-weaning. As the results shown, LH-LFD and HH-LFD gained more body weight than LL-LFD (**Figure 3A**). But there was no significant difference between HH-LFD and LH-LFD (**Figure 3A**). Nonetheless, there were no changes in GTT among three groups (**Figure 3C**). After 7 w HFD challenge, LH-

TABLE 2 | Primer sequences for RT-qPCR.

Gene	Forward sequence	Reverse sequence
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
Pgc-1α	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA
Adrb3	GGCCCTCTCTAGTTCCCAG	TAGCCATCAAACCTGTTGAGC
β-actin	AGAGGGAAATCGTGCGTGACA	CACTGTGTTGGCATAGAGGTC

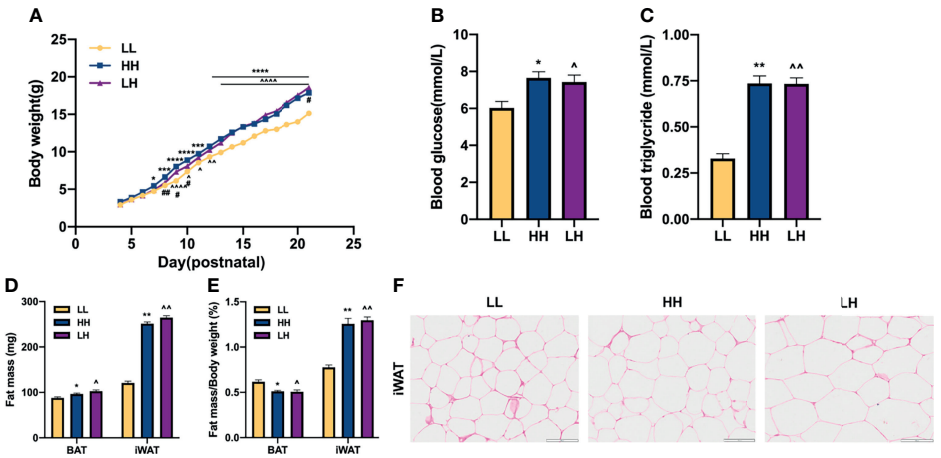

FIGURE 2 | Exposure to maternal HFD both prenatal and early postnatal or early postnatal alone predisposes the offspring for metabolic disorders at P21. **(A)** Postnatal daily changes in body weight of mice from the LL, HH, and LH group (n = 10, from 5 to 8 litters). Blood glucose **(B)**, Serum triglyceride **(C)** of offspring at weaning. **(D)** Fat tissue weight (BAT, brown adipose tissue; iWAT, inguinal adipose tissue) and **(E)** ratio of fat tissue to body weight of offspring at weaning (n = 3, from 3 litters). **(F)** Hematoxylin and eosin-stained iWAT sections of offspring at weaning (n = 3, from 3 litters). Data are expressed as the mean ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001: HH versus LL group. $^{\wedge}p$ <0.05, $^{\wedge\wedge}p$ <0.01, $^{\wedge\wedge\wedge\wedge}p$ <0.0001: LH versus LL group. $^{\#}p$ <0.05, $^{\#\#}p$ <0.01: LH versus HH group. Scale bar, 100 μm **(F)**.

HFD and HH-HFD had severely increased body weight (**Figure 3B**). We also noticed that the AUC of GTT in LH-HFD was nearly 0.5-fold increased than LL-HFD, and the AUC of GTT in HH-HFD was higher than LL-HFD (**Figure 3D**). Histological analysis of BAT morphology revealed the presence and accumulation of multiple and large lipid droplets in LH-LFD and LH-HFD (**Figure 3E**). Similar to BAT, LH-LFD and LH-HFD displayed largest lipid droplets in iWAT (**Figure 3F**). As evidenced by increased emergency of larger adipocytes, with exposure to maternal HFD both prenatal and early postnatal or early postnatal alone caused offspring obesity susceptibility. Additionally, LFD feeding from weaning to adulthood could not reverse such metabolic outcomes. Exposure to maternal HFD pre-weaning alone were most prone to developing obesity and metabolic disorder whether exposed to LFD or HFD diet at post-weaning.

Function of BAT Was Impaired in Adult Offspring of LH and HH, But LH Seemingly More Badly

BAT, as a major thermogenic center, plays an important role in energy metabolism and against obesity. Therefore, we detected the changes of BAT function (27). There was a noticeable histological difference in BAT among LL-LFD, HH-LFD, and LH-LFD. The LH-LFD had bigger adipocytes with largest lipid droplets and least Uncoupling protein 1 (UCP1) positive area. The UCP1 positive area of HH-LFD is less than LL-LFD (**Figure 4A**). Consistent with LFD, LH-HFD had least UCP1 positive area compared with HH-HFD and LL-HFD (**Figure 4A**). As the impairment of BAT activity in adult offspring, β3-adrenergic signaling pathway was examined. The relative mRNA expressions of thermogenic genes (Ucp1, Pgc-1a, Prdm16, and Cidea) and β3-adrenergic receptor (Adrb3) were decreased in the LH-LFD and HH-LFD, while LH-LFD presented a lowest expression of these markers among three groups (**Figure 4B**). In agreement, LH-HFD and HH-HFD presented downregulations of those relative mRNA expressions compared with LL-HFD, whereas the mRNA expressions of LH-HFD were lowest (**Figure 4C**). Next, we determined the expressions of relative proteins. When fed post-weaning LFD, LH-LFD displayed lowest UCP1 levels in BAT compared with HH-LFD and LL-LFD, and HH-LFD exhibited a lower UCP1 expression in BAT than LL-LFD (**Figure 4D**). Similarly, when exposed to post-weaning HFD, protein expressions of UCP1 were decreased in BAT of LH-HFD and HH-HFD as compared with LL-HFD. But LH-HFD showed lowest UCP1 level among three groups (**Figure 4E**).

Hormone-sensitive lipase (HSL) activity is essential for fatty acid oxidation, as a key effector of β3-adrenergic signaling (28). We further tested HSL levels in iWAT. When fed post-weaning LFD, LH-LFD and HH-LFD displayed downregulation of HSL phosphorylation in iWAT, and LH-LFD showed lower than HH-LFD. The ratio of p-HSL/HSL of LH-LFD was lowest in three groups (**Figure 4F**). UCP1 level of iWAT was consistent with the ratio of p-HSL/HSL (**Figure 4F**). Similarly, when exposed to post-weaning HFD, ratio of p-HSL/HSL in iWAT of LH-HFD and HH-HFD were markedly decreased, but ratio of p-HSL/HSL of LH-HFD was lowest (**Figure 4G**). UCP1 protein level of iWAT resembled the ratio of p-HSL/HSL (**Figure 4G**).

Together, the above results suggest that pre-weaning exposure to maternal HFD impaired the development of BAT in adult offspring both exposed to LFD and HFD at post-weaning. Furthermore, the offspring exposure to maternal HFD pre-weaning alone were most severely affected through inhibition of β3-adrenergic signaling.

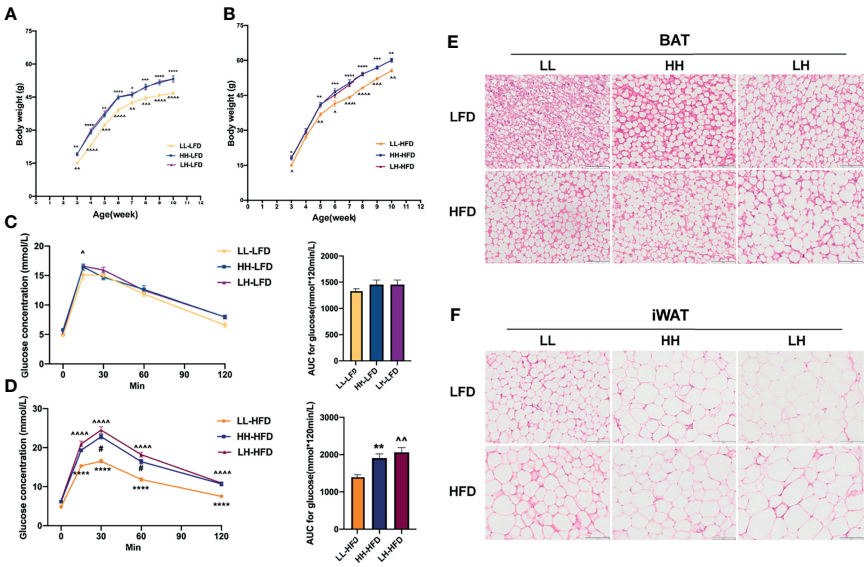

FIGURE 3 | Offspring of LH were most prone to developing obesity and metabolic disorder when challenged with HFD post-weaning. Body Weight changes of mice from the LL, HH, and LH group fed post weaning LFD **(A)** (n = 10, from 5 litters) or HFD **(B)** (n =10, from 5 litters) until they were 10 weeks old. GTT and AUC quantification of 10-week-old LL, HH and LH mice fed LFD **(C)** or HFD **(D)** (n = 5, from 5 litters). Hematoxylin and eosin–stained BAT **(E)** and iWAT **(F)** sections of 10-week-old LL, HH and LH mice fed LFD or HFD (n = 3, from 3 litters). Data are expressed as the mean ± SEM. **p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001: HH versus LL group. ^*p* <0.05, ^^*p* <0.01, ^^^*p* <0.001, ^^^^*p* <0.0001: LH versus LL group. #*p* <0.05: LH versus HH group. Scale bar, 100 μm **(E, F)**.

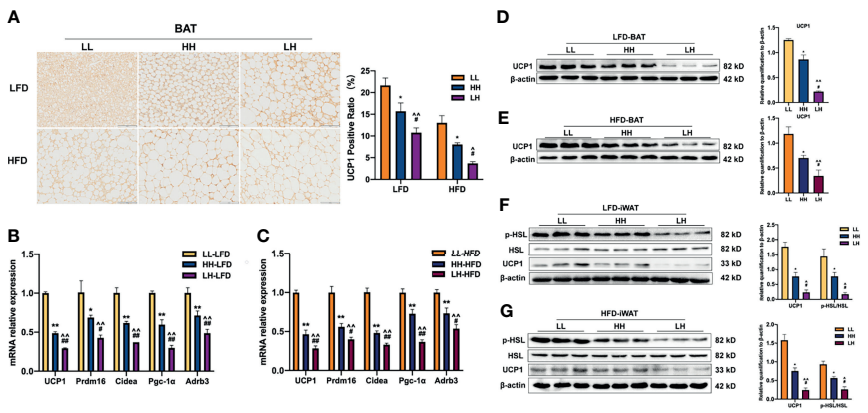

FIGURE 4 | Function of BAT was impaired in adult offspring of LH and HH, but LH seemingly more badly. **(A)** UCP1 staining for BAT sections of 10-week-old LL, HH and LH mice fed LFD or HFD (n = 3, from 3 litters) and UCP1 positive area. mRNA expressions of thermogenic genes in BAT when fed LFD **(B)** or HFD **(C)** (n =3, from 3 litters). Western blot analysis for thermogenic marker (UCP1) in BAT of adult LL, HH, and LH mice fed LFD **(D)** or HFD **(E)** (n =3, from 3 litters). Western blot analysis for UCP1, p-HSL and HSL in iWAT of adult LL, HH, and LH mice fed LFD **(F)** or HFD **(G)** (n = 3, from 3 litters). The fold increase was determined and normalized to β-ACTIN. Data are expressed as the mean ± SEM. **p* <0.05, ***p* <0.01: HH versus LL group. ^*p* <0.05, ^^*p* <0.01: LH versus LL group. #*p* <0.05, ##*p* <0.01: LH versus HH group. Scale bar, 100 μm **(A)**.

Exposure to Maternal HFD Pre-Weaning Alone Impaired the Adaptive Thermogenic Function in BAT of Adult Offspring Through SNS

To examine the long-term effect on adult offspring in BAT-mediated nonshivering thermogenesis, adult offspring received intraperitoneal injection of NE for activating sympathetic pathway. The control groups received the same dose of GS intraperitoneal injection, and the rectal temperature was measured. Before injection of NE/GS, rectal temperature of offspring was maintained at about 37°C. Intraperitoneal injection of GS did not markedly influence the rectal temperature.

After injected with NE, the rectal temperature began to rise. As the AUC for rectal temperature shown, HH-LFD and LH-LFD rectal temperature increased less than LL-LFD. Meanwhile, increased rectal temperature of LH-LFD was lowest (**Figure 5A**). When challenged with HFD at post-weaning, offspring presented similar variation to LFD after given NE. The increased rectal temperature of LH-HFD and HH-HFD were obviously lower than LL-HFD, but the rectal temperature increasement of LH-HFD was lowest (**Figure 5B**). There was no significant difference in rectal temperature when injected with GS.

After intraperitoneal injection of NE, by performing UCP1 immunohistochemistry we noticed larger adipocytes with less UCP1 staining in BAT of both LH-LFD and HH-LFD (**Figure 6A**). As compared with HH-LFD, however, LH-LFD BAT presented larger adipocytes and less UCP1 positive area (**Figure 6A**). When fed post-weaning HFD, adult offspring presented the same variation to LFD after received NE intervention (**Figure 6A**). Similarly, LH-LFD and HH-LFD possessed less UCP1-expressing but larger unilocular adipocytes in iWAT after intraperitoneal injection of NE compared with LL-LFD. LH-LFD presented larger unilocular adipocytes and less UCP1 positive area in iWAT than HH-LFD (**Figure 6B**). When fed post-weaning HFD, the largest adipocytes with least UCP1 stanning were detected in iWAT of LH-HFD compared with HH-HFD and LL-HFD, while HH-HFD presented larger lipid droplets and less UCP1 positive area than LL-HFD (**Figure 6B**).

Impaired adaptive thermogenic response in adult offspring of LH was subsequently confirmed by gene expression of several critical elements for thermogenesis. When fed post-weaning LFD, NE-induced Ucp1, Pgc-1a, Prdm16, Cidea and Adrb3 mRNA expressions showed remarkable reduction in BAT of LH-LFD and HH-LFD (**Figure 6C**), suggesting a low activation of BAT thermogenesis. In contrast to HH-LFD, the downregulation of those mRNA levels was more obvious in LH-LFD (**Figure 6C**). When fed post-weaning HFD, the similar change tendency was found (**Figure 6D**). Next, we investigated the protein levels of iWAT after receiving NE intraperitoneal injection. When fed post-weaning LFD, UCP1 level and ratio of p-HSL/HSL presented downregulation of LH-LFD and HH-LFD (**Figure 6E**). LH-LFD had lower UCP1 and p-HSL protein expressions than HH-LFD (**Figure 6E**). When fed post-weaning HFD, upregulation of UCP1 or p-HSL levels in response to NE stimulation in comparison with LL-HFD was not detected in LH-HFD and HH-HFD. But UCP1 and p-HSL levels in iWAT of LH-HFD were relatively lower than HH-HFD (**Figure 6F**).

Collectively, all these results demonstrated that the NE-induced adaptive thermogenesis in BAT was impaired in LH and HH adult offspring. Offspring from LH dams both fed post-weaning LFD and HFD presented severely impairment of BAT adaptive thermogenesis.

DISCUSSION

The intrauterine and early postnatal environments are critical in the development of offspring. Previous studies have demonstrated that altered maternal metabolism before delivery and during lactation have long-term effects on the future health

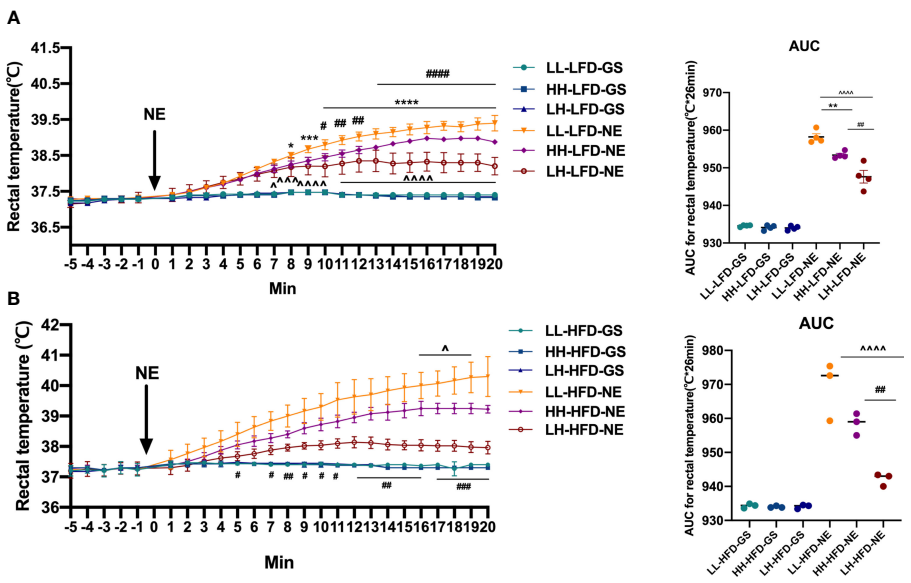

FIGURE 5 | Changes in rectal temperature of adult offspring after injection of NE/GS. Changes in rectal temperature of adult LL, HH, and LH mice fed LFD **(A)** or HFD **(B)** in 20 min after receiving NE or GS intraperitoneal injection (n = 4, from 4 litters). Data are expressed as the mean ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001, $^{****}p$ <0.0001: HH versus LL group. $^{\wedge}p$ <0.05, $^{\wedge\wedge}p$ <0.01, $^{\wedge\wedge\wedge}p$ <0.001, $^{\wedge\wedge\wedge\wedge}p$ <0.0001: LH versus LL group. $^{\#}p$ <0.05, $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001, $^{\#\#\#\#}p$ <0.0001: LH versus HH group.

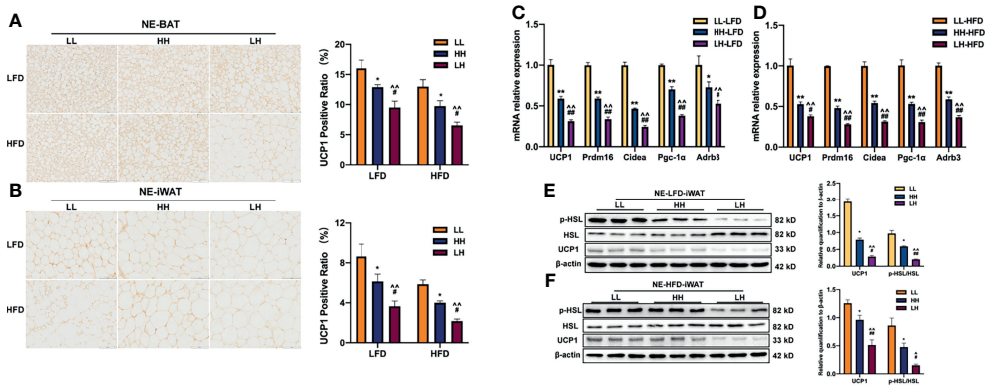

FIGURE 6 | Exposure to maternal HFD pre-weaning alone impaired the adaptive thermogenic function in BAT of adult offspring through SNS. UCP1 staining for BAT **(A)** or iWAT **(B)**sections of adult LL, HH and LH mice fed LFD or HFD (n = 3, from 3 litters) and UCP1 positive area after NE stimulation. mRNA expression of thermogenic genes in BAT when fed LFD **(C)** or HFD **(D)** (n = 3, from 3 litters). Western blot analysis for UCP1, p-HSL and HSL in iWAT of adult LL, HH, and LH mice fed LFD **(E)** or HFD **(F)** after NE stimulation (n = 3, from 3 litters). The fold increase was determined and normalized to β-ACTIN. Data are expressed as the mean ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01: HH versus LL group. $^{\wedge}p$ <0.05, $^{\wedge\wedge}p$ <0.01: LH versus LL group. $^{\#}p$ <0.05, $^{\#\#}p$ <0.01: LH versus HH group. Scale bar, 100 μm **(A, B)**.

prognoses of offspring (29, 30). In this study, we examined the offspring metabolic phenotype both at P21 and in adulthood. The results suggest that exposure to maternal HFD in both prenatal and early postnatal predisposes offspring for metabolic disorder, with implications for BAT thermogenic impairment. We further demonstrated the impairment of BAT thermogenesis was partially as a result of the attenuation of β3-adregernic signaling. In addition, our study extended these observations and demonstrated that the pre-weaning period was more sensitive to the altered developmental environment in response to maternal HFD. This finding is in agreement with previous reports demonstrating that an altered milieu at pre-weaning environment in rodents is sufficient to predispose for metabolic disturbances and can partly override prenatal susceptibility factors and genetic predisposition to develop obesity (31, 32). More importantly, our studies provide the evidence that pre-weaning HFD results in permanent changes in BAT thermogenesis through the sympathetic nervous system mediated mechanism.

In this study, the rodent mice model was used to dissociate the pre- and postnatal effects of maternal diet-induced or inherited obesity. We detected maternal metabolic phenotype in our previous study, and we found that mice exposed to HFD diet before mating responded with increased body weight, adiposity, and insulin resistance. But there was no significant difference in blood glucose and glucose tolerance test (GTT) between LFD and HFD dams before mating, indicating HFD that caused maternal obesity but did not impair glucose metabolism. Our study showed that maternal HFD throughout gestation and lactation resulted in offspring with increased body weight, adiposity, impaired glucose tolerance and thermogenesis of BAT at P21, and also greater susceptibility to diet-induced obesity in adulthood. Furthermore, offspring from maternal HFD at pre-weaning alone were most vulnerable to metabolic abnormalities at P21. Therefore, the metabolic alterations in HH and LH offspring might not be due to maternal pregestational dysmetabolism but an alteration of maternal diet composition and the changes of breast milk components, which was consistent with previous studies (33, 34). Additionally, as a consequence of pre-weaning exposure to maternal HFD, we noticed the increments in adipocytes diameter in iWAT and reduction of multilocular lipid droplets in BAT in offspring at P21 (results not shown). This strongly implicated that offspring obesity was related to the impairment of BAT thermogenic function. As the birth weight is a key pregnancy outcome related to metabolic health in adult life, subsequently, we investigated the phenotypic plasticity and assess their susceptibility to metabolic dysfunction. After 7 w HFD challenge, LH-HFD and HH-HFD had severely increased in body weight. We also noticed that the AUC of GTT in LH-HFD and HH-HFD was higher than LL-HFD. The body weight and GTT of offspring receiving LFD post-weaning corresponded with those that received HFD. Apparently, offspring maintained this metabolic phenotype changes in adulthood. It is conceivable that in our study, exposure to maternal HFD at pre-weaning could lead to obesity and glucose dysmetabolism and increased susceptibility to metabolic abnormalities provided with HFD post-weaning.

BAT is mainly consists of abundant mitochondria and multilocular lipid droplets. As a critical energy metabolism organ, BAT dissipates energy by means of uncoupled respiration and heat production (35). Nonshivering thermogenesis, a physiological process, is regulated by UCP1 and activated by cold exposure, adrenergic compounds, and genetic alterations. WAT can store excess energy as triglycerides (TG) which can be released as free fatty acids (FFAs) to participate in the β-oxidation to meet the energy demand, which is a process called lipolysis (35–38). Therefore,

promotion of BAT activity increases energy expenditure and protects against obesity and type 2 diabetes (35). Histologically, in adulthood, we noticed the larger lipid droplets in iWAT and BAT of LH-HFD. This morphology alteration was similar to early postnatal period, which was further exaggerated in offspring exposed to HFD post-weaning compared with LFD. In adulthood, transcription levels of UCP1 and other BAT marker gene Prdm16, Pgc1α, Cidea were downregulated in BAT of offspring from HH and LH dams, suggesting there were defects in BAT function. It was further confirmed by the fact that NE-induced rectal temperature elevation was attenuated in BAT of HH and LH offspring. Those changes were more obvious of offspring from LH dams, which indicated that offspring exposure to maternal HFD pre-weaning alone were more prone to develop metabolic disorder. Adaptive thermogenesis and BAT UCP1 expression are under the strict control of the sympathetic nervous system (SNS). Activation of sympathetic nerves by cold exposure results in the release of norepinephrine (NE). Combination of NE and β-adrenergic receptors (primarily the β3-adrenoceptor) leads to the activation of cAMP-dependent protein kinase (PKA) and the phosphorylation of HSL. FFAs from lipolysis or circulation are utilized as UCP1 substrates of β-oxidation in BAT mitochondria. The transcription of UCP1 uncouples respiration from the ATP synthesis and thus induces heat generation (39–41). Precious studies have shown that Adrb3 played a major role in thermogenesis in rodents, so we detected the transcription level of Adrb3 in BAT. And there was an obviously decline in Adrb3 mRNA level in offspring from HH and LH dams in adulthood, reflecting the decreased sympathetic innervation. To further examine the BAT function, adult offspring received NE injection to induce thermogenesis. We discovered the similar tendency after NE intervention, that offspring from LH dams showed lowest rectal temperature rise, indicating pre-weaning HFD impaired the adaptive thermogenesis. We also found the significant downregulation of UCP1 and attenuation of p-HSL activation with reduction of BAT UCP1 positive area in adulthood. This finding, moreover, confirm that pre-weaning HFD as being possible candidates inducing further changes of downstream autonomic pathways, potentially impairs the regulation of energy and glucose homeostasis in the offspring.

In our study, we established an animal model to detect the metabolic phenotype and BAT thermogenic function in P21 and adulthood periods, and demonstrated the effects of maternal before delivery and lactation nutrition status on offspring. But the underlying mechanism remains obscure. Leptin has been shown to be a critical factor in the development of hypothalamic feeding circuitry, which was related to the energy homeostasis (42–44). In other studies of our team, we explored the possible reason of abnormal hypothalamic feeding circuit in the case of maternal overnutrition stress. We have determined that the early hypothalamic supraphysiological stress response may lead to defective postnatal ARH-PVH neuropeptidergic projection in offspring exposed to maternal obesity and is related to impaired leptin signaling. Our study suggests that attenuation of BAT thermogenesis may be a key mechanism linking pre-weaning HFD to persistent metabolic abnormality in the offspring (26). Further studies will focus on identifying the mechanisms involved in correction of high fat diet-related deficits by manipulating postnatal diet or behavior (such as exercise) and refining the critical windows for development of systems regulating energy homeostasis and associated metabolic processes. In this study, our results reveal a critical timing for the development of BAT, and the pre-weaning period might be a window of susceptibility where intervention is possible.

CONCLUSIONS

Our study confirmed and extended previous observations showing that maternal HFD at pre-weaning predisposes offspring for metabolic disorder with implication for BAT thermogenic function, which was partially due to attenuation of β3-adregernic signaling. Furthermore, the pre-weaning period was more sensitive to maternal HFD. Our results provide clear evidence that exposure to maternal HFD during pre-weaning period alone showed similar detrimental effects on programming metabolic system of offspring as those of both prenatal and early postnatal HFD feeding. Pre-weaning is a critical window for metabolic programming and has profound and long-lasting effects on BAT development and function through sympathetic nerve-mediated thermogenesis. The next challenge will probably focus on activating BAT thermogenesis in adult humans to increase whole-body energy expenditure, lose weight and prevent T2DM.

STUDY LIMITATIONS

The present study was carried out only with male offspring mice. However, how pre-weaning exposure to maternal HFD causes adverse consequences and its specific mechanisms *in vivo* are still not completely known. In addition, maternal physiological changes caused by exposure to HFD before delivery may also invade pregnancy and lactation, and affect the development and growth of fetus or newborn through various factors. This is our current deficiency, but also our next work direction. The follow-up research will focus on HL (maternal HFD before delivery but LFD during lactation) mothers, in order to further improve the comprehensive exploration of the impact of maternal factors on 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.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Management Committee of the First Affiliated Hospital of Chongqing Medical University (Approval No- 2021-02).

AUTHOR CONTRIBUTIONS

HY, NC, and XqX contributed to conception and design of the study. HY conducted experimental research and wrote the first draft of the manuscript. NC organized the database. XjL revised the manuscript. FL, JcL and YhY performed the statistical analysis. YZ and YC contributed to Conceptualization. JbL contributed to review, editing and Supervision of the study. XqX supervised the experimental process, put forward opinions and revised the manuscript. All authors contributed to manuscript revision, read, 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/fendo.2022.816107/full#supplementary-material

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Lipidomic Analysis of TRPC1 Ca^{2+}-Permeable Channel-Knock Out Mouse Demonstrates a Vital Role in Placental Tissue Sphingolipid and Triacylglycerol Homeostasis Under Maternal High-Fat Diet

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The transient receptor potential canonical channel 1 (TRPC1) is a ubiquitous Ca^{2+}-permeable integral membrane protein present in most tissues, including adipose and placenta, and functionally regulates energetic homeostasis. We demonstrated that elimination of TRPC1 in a mouse model increased body adiposity and limited adipose accumulation under a high fat diet (HFD) even under conditions of exercise. Additionally, intracellular Ca^{2+} regulates membrane lipid content *via* the activation of the protein kinase C pathway, which may impact placental membrane lipid content and structure. Based upon this we investigated the effect of HFD and TRPC1 elimination on neutral lipids (triacylglycerol and cholesteryl ester), membrane lipids (phosphatidylcholine and phosphatidylethanolamine), and other multifunctional lipid species (unesterified cholesterol, sphingomyelins, ceramides). The concentration of unesterified cholesterol and sphingomyelin increased with gestational age (E12.5 to E 18.5.) indicating possible increases in plasma membrane fluidity. Diet-dependent increases ceramide concentration at E12.5 suggest a pro-inflammatory role for HFD in early gestation. TRPC1-dependent decreases in cholesterol ester concentration with concomitant increases in long-chain polyunsaturated fatty acid -containing triacylglycerols indicate a disruption of neutral lipid homeostasis that may be tied to Ca^{2+} regulation. These results align with changes in lipid content observed in studies of preeclamptic human placenta.

Keywords: infusion lipidomics, placental lipidome, TRPC1, sphingolipid metabolism, triacylglycerol

INTRODUCTION

High fat diet (HFD)-fed mothers and fathers are obese, hyperglycemic, and hyperlipidemic (1). A parental HFD also contributes to gestational programing of offspring obesity (2–5). We have demonstrated that TRPC1 increases total body adiposity in mice by decreasing the efficacy of exercise to limit adipose accumulation under a HFD (6). Placental lipid regulation is vital to the

development of a healthy placenta and fetus. Obesity and a HFD are associated with increased risks of preeclampsia, gestational diabetes, placental inflammation, and fetal macrosomia (7–9). Even during healthy pregnancies, placentae from obese women contain 20% more esterified lipid than placental samples from non-obese women, which impairs placental function (10). Investigating mechanistic relationships among adiposity, placental function, and putative placental tissue lipid content-associated placental dysfunction are important for understanding how HFD-induced obesity may impact fetal development mediated *via* placental dysfunction.

We have demonstrated that adipose proliferation is reduced in mice by knocking-out transient receptor potential canonical channel 1 (TRPC1) (11). TRPC1, a ubiquitous member of the transient receptor potential superfamily, is an integral membrane protein that regulates Ca^{2+} ion flux across the membrane (12). We have demonstrated that TRPC1 -/- animals have reduced adipocyte differentiation, reduced markers for autophagy, and increased expression of apoptosis markers, suggesting a change in neutral lipid storage and the sphingomyelin-ceramide pathway regulating nutrient transport, cell proliferation, and apoptosis (13, 14). We also demonstrated lower adipose mass in HFD fed TRPC1 -/- mice when compared to HFD fed WT mice (11, 15). When provided access to exercise, HFD fed TRPC1 -/- mice experienced greater loss of adipose by mass and reduced insulin resistance compared to WT animals under the same conditions, indicating TRPC1 plays a role in systematic energy homeostasis, particularly neutral lipid metabolism.

The hydrolysis of membrane-bound sphingomyelin (SM) to generate the secondary messenger ceramide (Cer) is a highly conserved signaling pathway that responds to cellular stress and regulates apoptosis (13). We hypothesized that placentae from TRPC1 -/- dams have increased Cer concentration when compared to control animals due to the metabolic changes we have previously observed in this mouse model (11, 15). If this is the result of increased SM hydrolysis, then there should be a concomitant decrease in SM concentration. Using a HFD high in saturated fat, there should be an additive increase in Cer concentration (16). Additionally, we aimed to determine whether the absence of TRPC1 gene in placentae altered the composition of neutral lipid storage. To test this hypothesis, we developed a comprehensive infusion lipidomic workflow which measured Cer, SM, phosphatidylcholines (PC), phosphatidylethanolamines (PE), triacylglycerols (TAG), cholesterol esters (CE), and unesterified cholesterol (FC).

EXPERIMENTAL

Animal Protocol Design and Approval

Two-month-old female B6129SF2/J mice (Envigo, Indianapolis, IN) were fed diets containing either 16% (normal-fat, NF) or 45% fat (high-fat, HF) for 12 weeks (**Table S1**). Following 12 weeks of diet intervention, dams were bred with normal chow fed males and pregnancy identified by the presence of a vaginal plug. Midday identification of the vaginal plug was considered embryonic day 0.5 (E0.5). Dams were maintained on their respective diets throughout mating and pregnancy. Dam euthanasia was by CO_2 inhalation according to the animal use and care protocol approved by the USDA Agricultural Research Service, Grand Forks Human Nutrition Research Center Animal Care and Use Committee. Fetuses and placentae were harvested in mid-gestation (E12.5-E13.5) or late gestation (E18.5-E19.5), weighed, measured, and then immediately frozen in liquid nitrogen.

Placental and Fetal Tissue Weight Measurements

The uterine horn was dissected from the dam and cut between each implantation site separating each amniotic sac containing individual fetus. Removed placenta and fetus were blotted dry before removal of umbilical cords. Weights were recorded and the placenta length and width were measured with the maternal convex side up using a digital caliper (Marathon Watch Company LTD., Richmond Hill, ON Canada). Samples were then flash frozen in liquid nitrogen and stored at -80°C.

Placental Lipid Analysis

HPLC-grade isopropanol, chloroform, butylated hydroxytoluene, and hexane were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as received. HPLC-grade methanol was purchased from Honeywell (Muskegon, MI, USA). Silicic acid (200-325 mesh) was ordered from Clarkson Chromatography Products Inc. (South Williamsport, PA, USA). Internal standards for TAG and CE analysis were purchased from NuChek Prep Inc. (Elysian, MN, USA), phospholipid and sphingolipid standards and LIPIDMAPS standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA) (**Table S2**).

Briefly, frozen placental samples were homogenized in aqueous buffer. Neutral lipid internal standards were added, and lipids were extracted in 3:2 hexane: isopropanol (50 μmol/L BHT). Extracts were isolated and dried under, then redissolved in 1 mL chloroform. The solution was divided in half, with 500 μL retained for phospholipid analysis (below). Phospholipids were removed from the neutral lipid fraction by dispersive SPE with silicic acid, and100 μL of the supernatant was combined with 100 μL of methanol (20 mM ammonium acetate). This sample (Solution 1) was analyzed for TAG and CE content as described below. A 500 μL aliquot of the SPE supernatant was used for the assay of FC following the method of Liebisch et al. (17).

Analysis of polar lipids was performed using the method adapted from Sundaram et al. with modifications made to allow for automation (18).

Data Collection and Analysis

Data were collected on an AB Sciex 5500 QTRAP hybrid mass spectrometer equipped with a Turbo V electrospray ion source and SelexION ion mobility device (AB Sciex, Framingham, MA, USA). Samples were infused using a Shimadzu Prominence UPLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with an LC20XR autosampler (50 μL stainless steel sample loop) and two solvent delivery units

following a configuration modified from Bukowski and Picklo as detailed in the **Supplemental Information** (19).

Multiple instrument modes were used based upon lipid class and preparation (**Table S2**). TAG were characterized and quantitated by brutto structure as ammoniated cations, $[TAG + NH_4]^+$ in enhanced mass spectrum (EMS) mode as previously published (19, 20). Cholesterol esters (CE) were assayed by neutral loss scan for 20 fatty acid neutral losses with confirmatory detection of the cholesterol head group by product ion scan for mass-to-charge ratio (m/z) = 369, as previously published (**Table S3**) (20, 21). Acylated samples for FC determination were analyzed using multiple reaction monitoring for the acylated d7-cholesterol species (*m/z* = 453.4→376.3) and the endogenous acylated cholesterol (*m/z* = 446.4→369.3) (17). Ceramide species (Cer) were detected using the product ion m/z 264, selective to the sphingosine backbone, and quantified following the method of Picklo *et al.* (22). Phosphatidylethanolamine, and phosphatidylcholine species were quantified as brutto species using our previously published methods (20, 23). Monitoring the neutral loss of 141 Da allowed for the selective measurement of PE species, while monitoring the product ion m/z = 184 was selective for PC and SM species.

SM species were isolated for characterization and quantitation using the Selexion ion mobility device (24).

Mass spectra for all lipid class were examined manually to confirm brutto structure assignment. Isotopic and ionization correction factors were determined as previously described and quantitation of target species was performed using LipidView software (AB Sciex, Framingham, MA, USA) (19, 20, 22, 25). Values were normalized to tissue wet weight. TAG 54:0 and TAG 54:1 were excluded from analysis due to isobaric overlap with a silicone oligomer contaminant which was extracted from the septa and could not be reliably removed by subtraction.

Statistical Analysis

Statistical analyses were performed using MetaboAnalyst 5.0 (26). After range scaling, data were analyzed by one-way ANOVA with an alpha of P<0.05, and a 0.05 false discovery rate. Tukey's HSD was employed to as a *post-hoc* test for significant interactions between groups.

RESULTS

Types and Amount of Placental Lipid Determination

Using the combined infusion mass spectrometric methods 190 major lipid species including sphingolipids and phospholipids were identified in the placental samples. In the neutral lipid fraction 34 cholesterol esters, 50 TAG and FC were quantitated (additional details provided in **Tables S4, S5**). In the polar lipid fraction, brutto structures included 49 PC, 36 PE, eight SM, eight Cer, and four HexCer species (**Tables S6–S8**, respectively). A summary of the relationships established by univariate analysis is presented in **Table 1**.

Cer and other glycosphingolipids (cerebroside) species were identified based upon the m/z = 264 product ion which was selective for the sphingosine base, d18:1, allowing for identification of the fatty acid moiety based upon the precursor ion scan for the Cer species. As shown in **Figure 1**, PC and SM species were both detected in the precursor ion scan. The monoisotopic peaks used for quantitation of SM and PC species were offset one Dalton and presented overlap as in the case of the grouping of peaks from m/z 806-816, in which species PC 38:4, PC 38:3, PC 38:2, and PC 38:1 overlapped with SM 42:3, SM 42:2, and SM 42:1. Removal of PC the contribution using the Selexion ion mobility interface was necessary to accurately quantitate to SM, which represented approximately 4% of the signal. A difference in acyl carbon distribution between PC and PE species was evident (**Figure 1**).

When compared, PE and PC species with equivalent acyl carbon numbers in placental membrane phospholipids demonstrated an increase in concentration of LC-PUFA moieties in PE (**Figure 1**, dashed lines). The majority of PC species had 34, and 36 acyl carbons with between 0 and 3 double bonds, corresponding to structures containing 16- and 18-

TABLE 1 | Summary of lipid classes and relationships observed by 1-way ANOVA (p < 0.05) with.

Relationship		Lipid Class								
		FC	CE	TAG	PC	PE	SM	CER	HexCer	Total
WT -HF-M vs. WT -HF-L	Gestation	1	1	3	1	1	7	3	0	17
WT -NF-M vs. WT -NF-L	(*)	1	10	4	1	1	7	0	1	25
TRPC1 -/- -HF-M vs. TRPC1 -/- -HF-L		1	14	2	14	10	7	3	0	51
TRPC1 -/- -NF-M vs. TRPC1 -/- -NF-L		1	0	2	2	0	6	7	1	19
TRPC1 -/- -NF-L vs. TRPC1 -/- -HF-L	Diet	0	15	0	0	0	7	0	0	22
TRPC1 -/- -NF-M vs. TRPC1 -/- -HF-M	(**)	0	0	4	10	2	3	7	0	26
WT -NF-L vs. WT -HF-L		0	3	3	2	0	3	5	0	16
WT -NF-M vs. WT -HF-M		0	24	1	1	0	1	8	0	35
WT -HF-L vs. TRPC1 -/- -HF-L	Genotype	0	1	15	0	0	1	0	0	17
WT -HF-M vs. TRPC1 -/- -HF-M	(‡)	0	10	0	5	4	1	1	0	21
WT -NF-L vs. TRPC1 -/- -NF-L		0	6	9	1	0	3	4	0	23
WT -NF-M vs. TRPC1 -/- -NF-M		0	0	1	0	0	0	0	0	1

Symbols indicate differences with p<0.05 base upon one-way ANOVA with Tukey's HRD post hoc test and application of a 0.05 false discovery rate for gestational age (*), diet (**), and genotype (‡). P-values are available in ***Supplemental Materials****. WT, wild-type; TRPC1 -/-, TRPC1 knock-out; NF, normal fat diet; HF, high-fat diet, M, mid-gestation; L, late gestation.*

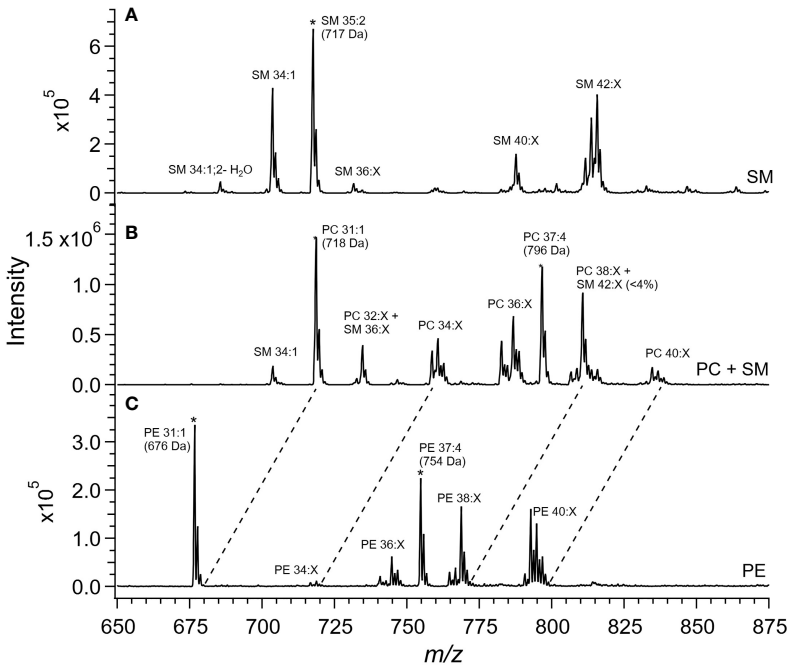

FIGURE 1 | Mass spectra of placental extracts from NF-WT mouse. Selective scans for **(A)** sphingomyelin (SM) using the product ion *m/z* 184 with differential ion mobility, **(B)** phosphatidylcholine and sphingomyelin using product ion *m/z* 184, and **(C)** phosphatidylethanolamine using the neutral loss of 141 Da. Internal standards are indicated with *. Dashed lines indicate glycerophospholipid groups with common acyl carbon number.

carbon fatty acids such as palmitic, palmitoleic, stearic, oleic, and linoleic acids while the dominant PE species had 40 acyl carbons and between 4 to 7 double bonds and were composed largely of PUFA species as detailed in the **Supplemental Information** (**Table S9**) (25).

Lipid Content and Species Distribution Was Affected by Gestational Age

The gestational age of the placenta affected the neutral lipid and sphingolipid content. For neutral lipid species the concentration of FC (**Figure 2A**) increased from mid-gestation to late-gestation irrespective of diet or genotype. While animals on HFD appeared to have greater FC at mid gestation, biological variability rendered this difference non-significant and placental FC concentrations at late gestation were indistinguishable. A gestational age-dependent increase in total CE concentration was observed for WT-NF and TRPC1 -/- -HF animals. For the WT-NF group the increase was due primarily to increases in major species such as CE 16:0, CE 18:0, CE 18:1, CE 18: 2, (**Figure 3A**), though gains in CE 16:1, CE 20:2, CE 22:5, CE 22:6 and CE 24:1 also contributed to the elevated concentration. The major contributor to the increase in CE concentration for TRPC1 -/- -HF animals was CE 20:4, which increased 1.9-fold from mid to late gestation. Gestation-dependent increases in CE 22:5 (4.3-fold, **Figure 3B**) and CE 22:6 (2.9-fold, **Figure 3B**) and CE 22:4 (2.6-fold, **Figure 3A**) were also observed. These trends were not observed for either the TRPC1 -/- -NF or WT-HF animals, though the latter did exhibit a decrease CE 16:1 concentration (**Figure 3B**).

Total placental TAG concentration was not affected by gestational age; however, individual TAG species underwent gestation-dependent changes. Tissue from WT-HF dams demonstrated increased concentrations of TAG 50:1 and TAG 48:0 with a concomitant decrease in TAG 56:7 (**Figures 4A, B**). For WT animals, irrespective of diet, the most concentrated TAG species, (TAG 54:2, TAG 54:3, TAG 56:6, TAG 56:8, TAG 58:6, TAG 58:8) were stable over the observed gestational time scale, however TRPC1-knockout animals experienced decreases in TAG 58:6 and TAG 58:6 (**Figures 4B, C**), though the concentrations of these species were higher at the early gestational time point, as will be discussed below.

Gestational age affected the concentration of sphingolipids in placentae. In all cases the SM concentration increased with gestational age (**Figure 2B**). The major contributors to the net increase in SM concentration were SM 42:1, SM 42:2, and SM 34:1 (**Figure 5B**). Total Cer concentrations were unchanged for WT-NF samples but increased with gestational age for TRPC1 -/- -NF (**Figure 2B**). Cer d18:1/16:0, Cer d18:1/22:0, Cer d18:1/24:0, and Cer d18:1/24:1 were the major contributors to this increase (**Figure 5A**) Contrastingly, the WT-HF and TRPC1 -/- -HF arms exhibited decreased Cer concentration with increasing gestational age, as well as a decrease in total HexCer concentration.

Total concentration PE species were not affected by gestational age in aggregate (**Figure S1**); however, PE 40:4 decreased in concentration for nearly all groups with increasing gestational age (**Figure S2B**). Only placentae from TRPC1 -/- -HF dams demonstrated a loss in PC concentration (**Figure S1**) but the

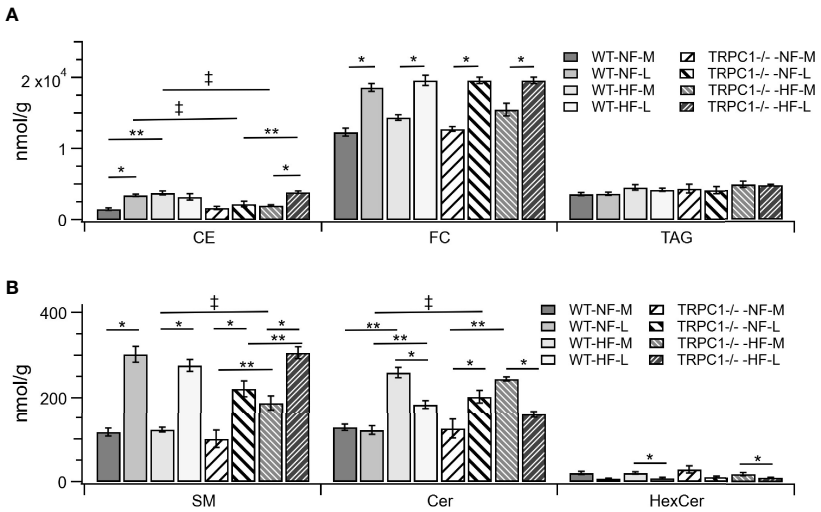

FIGURE 2 | Lipid class differences between groups for **(A)** cholesterol esters (CE), unesterified cholesterol (FC), triacylglycerols (TAG). Sphingolipids are shown in **(B)**, sphingomyelin (SM), ceramide (Cer), and hexosylceramides (HexCer). Data are shown as mean ± sem (n = 8, except for TRPC1 -/- -HF-M where n = 4). Symbols indicate differences with p<0.05 base upon one-way ANOVA with Tukey's HRD *post hoc* test and application of a 0.05 false discovery rate for gestational age (*), diet (**), and genotype (‡). WT, wild-type; TRPC1 -/-, TRPC1 knock-out; NF, normal fat diet; HF, high-fat diet; M, mid-gestation; L, late gestation.

concentration major contributors to that change were PC 36:2, PC 36:3, PC 38:3, and PC 34:2 (**Figures S3A, B**).

Lipidomic Responses to HFD Indicate Difference in Neutral Lipid Storage Between WT and TRPC1 -/- Placentae

Cholesterol ester concentrations at the earlier gestational time point were greater (p<0.05) in placentae from HFD fed dams irrespective of phenotype (**Figure 2A**). Species-level univariate analysis revealed the diet-induced increase in cholesterol ester concentration was independent of fatty acid identity, with increases in concentration for nearly all cholesterol ester species (**Figure 3**). The exceptions to this trend were CE 16:0 and CE 16:1, which demonstrated decreases from WT-NF -L to WT-HF-L. Tissue from TRPC1 -/- -HF-L dams demonstrated increases in CE 18:2, CE 20:4, CE 22:4, CE 20:3 CE 22:5, and CE

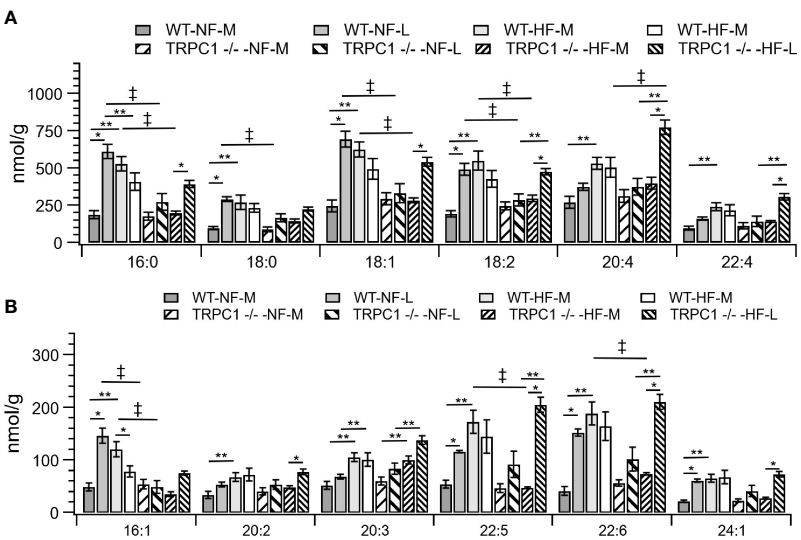

FIGURE 3 | Placental CE concentration by species for CE C:N. C = acyl carbon number and N = acyl desaturation level. Species are grouped with concentration decreasing from **A** to **B** to demonstrate the range of concentrations. Data are shown as mean ± sem (n = 16, except for HF-TRPC1 where n = 12). Symbols indicate differences with p<0.05 base upon one-way ANOVA with Tukey's HRD *post hoc* test and application of a 0.05 false discovery rate for gestational age (*), diet (**), and genotype (‡). P-values are available in **Supplemental Materials**. WT, wild-type; TRPC1 -/-, TRPC1 knock-out; NF, normal fat diet; HF, high-fat diet, M, mid-gestation; L, late gestation.

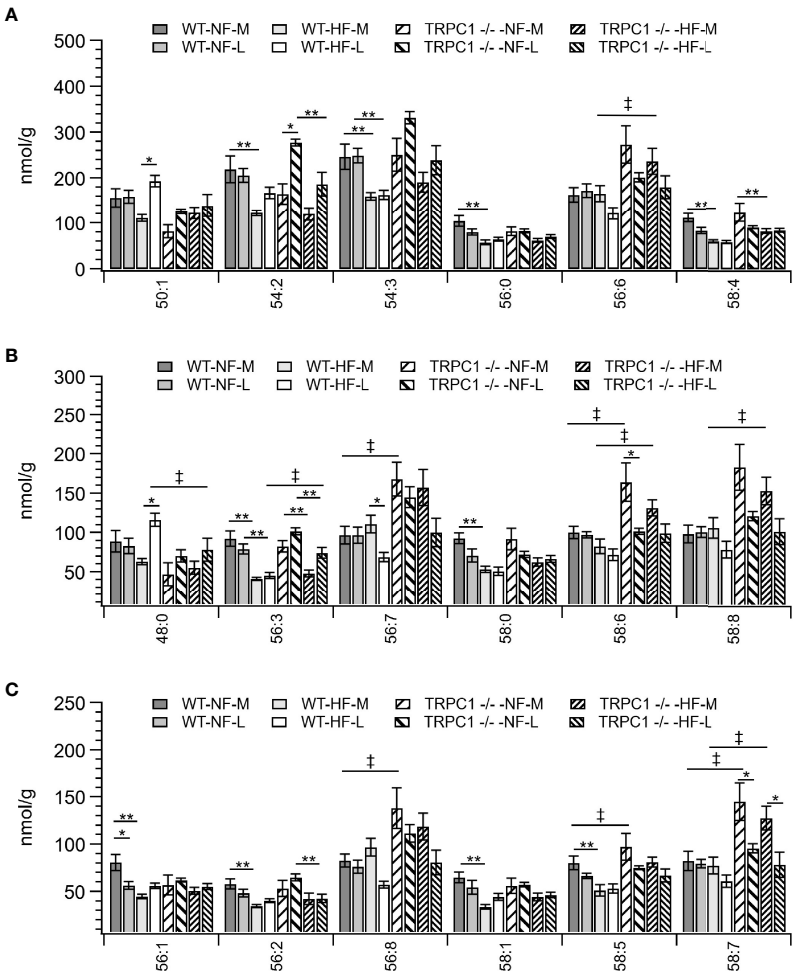

FIGURE 4 | Placental TAG concentration by species for TAG C:N. C = acyl carbon number and N = acyl desaturation level. Species are grouped with diminishing concentrations from **A–C** to illustrate the range of concentrations. Data are shown as mean ± sem (n = 16, except for HF-TRPC1 where n = 12). Symbols indicate differences with p<0.05 base upon one-way ANOVA with Tukey's HRD *post hoc* test and application of a 0.05 false discovery rate for gestational age (*), diet (**), and genotype (‡). P-values are available in **Supplemental Materials**. WT, wild-type; TRPC1 -/-, TRPC1 knock-out; NF, normal fat diet; HF, high-fat diet; M, mid-gestation; L, late gestation.

22:6 relative to TRPC1 -/- -NF-L though, with the exception of CE 20:4, the concentrations at this time point were not significantly different from WT-HF-L samples.

Differences in genotypic response to HFD were observed between the WT-HF-M and TRPC1 -/- -HF-M arms (**Figures 3A, B**). At the earlier time point the SFA-containing species CE 16:0 and CE 18:0 were only 37% and 53% of the WT-HF-L arm, respectively. This trend held for MUFA-containing CE (CE 18:1 and CE 16:1; 53% and 30% of WT-HF-L, respectively), as well as PUFA-containing CE species such as CE 18:2, CE 20:5, CE 22:5, and CE 22:6 (54%, 54%, 28%, and 39% of WT-HF-L, respectively). These differences were not present at the later time point for the HFD arms. These data, along with the observed increase in CE 20:4 noted earlier suggests a compensatory mechanism for PUFA CE accumulation.

For samples from WT animals consumption of HFD led to a decrease in TAG storage for TAG 54:2, 54:3, 54:7, 54:9, 56:0, 56:1, 56:2, 56:3, 56:9, 56:10, 58:0, 58:1,58:2, 58:4, 58:5, 58:10, 58:11, 60:11, and 60:12 (**Figures 4A–C** and **Figure S2**). TAG species which displayed genotypic responses followed the pattern of presenting elevated TAG levels for the TRPC1 -/- placentae relative to the WT at the earlier gestational time point. These species included TAG 56:3, 56:6, 56:7, 56:8, 58:5, 58:6, 58:7, 58:8, 58:10, 60:11, and 60:12 (**Figures 4A–C** and **Figure S2**). TAG 48:0 presented as an outlier to this trend presenting greater in WT-HF-L animals that the corresponding TRPC1 -/- arm (**Figure 4B**).

Sphingolipid Concentrations in Placental Tissue Were Altered by Gestation, Diet, and Genome

As discussed above, the general trend observed for SM species was an increase in concentration with gestational age irrespective of diet or genotype. For SM 34:1 a genotype-dependent response was observed. At the later gestation time point the concentration of SM 34:1 for TRPC1 -/- -NF-L was 64% of that observed for the

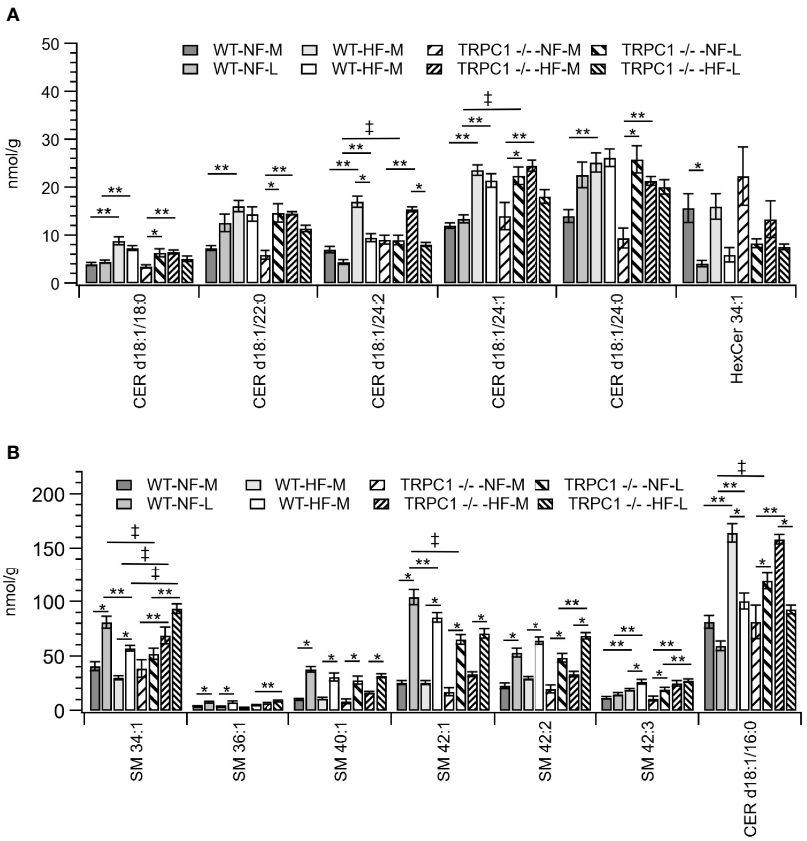

FIGURE 5 | Placental sphingolipid concentration by species. **(A)** Cer, and **(B)** SM species. Data are shown as mean ± sem (n = 8, except for HF-TRPC1-M where n = 4). Symbols indicate differences with p<0.05 base upon one-way ANOVA with Tukey's HRD *post hoc* test and application of a 0.05 false discovery rate for gestational age (*), diet (**), and genotype (‡). P-values are available in **Supplemental Materials**. WT, wild-type; TRPC1 -/-, TRPC1 knock-out; NF, normal fat diet; HF, high-fat diet; M, mid-gestation; L, late gestation.

corresponding WT-NF-L arm, however TRPC1 -/- -HF-M and TRPC1 -/- -HF-L had SM 34:1 concentrations that were 230% and 160% greater than in the corresponding WT groups (**Figure 5B**).

In addition to the gestational trends indicated above, the concentration of Cer species tended to be higher in placentae from WT-HF animals. For TRPC1 -/- -NF-L subjects the concentrations of Cer d18:1/16:0, Cer d18:1/24:1, and d18:1/24:1 increased with gestational age and were greater than corresponding WT-NF-L subjects (**Figure 5B**).

DISCUSSION

This is the first analysis of mouse placentae at two gestational time points that demonstrates the effect of HFD on lipidomic profiles. Our first measurement at E12.5 corresponds to the appearance of the first definitive placenta, while the second measurement point near E18.5 represents a fully functioning organ nearing the end of its lifespan, preparing for parturition (27, 28). The visibly greater concentration of FC between the two diet arms at the early time point was not present by E18.5. Perhaps a nutritional sensitivity for FC early in pregnancy diminishes as the placenta develops and regulates cholesterol homeostasis and cholesterol efflux to the fetus. This could in part explain why, in a rodent model, cholesterol supplementation of the maternal diet impacts the serum lipid profile (HDL, LDL, TG, total cholesterol) without altering the fetal lipid profile (29). and the decrease in serum lipid concentrations post parturition in humans (30). Increased FC may also reflect greater membrane fluidity during placental development (31). Future work evaluating the impact of HFD on the transcription of enzymes involved in the biosynthesis and transport of cholesterol such as DHCR-7, Abca1, Abcg1 and Sr-b1 would shed light on the underlying mechanisms of this observation.

Our CE results also indicate a role for the placenta in fetal lipid homeostasis under HF conditions. While the placentae from WT-NF dams increased in CE concentration with gestational age, under WT-HF conditions the CE concentration decreased with gestational age, particularly for CE 16:1. Palmitoleic acid is a marker for *de novo* lipogenesis (DNL) (32), and a HFD suppresses DNL (33), specifically for

lard-based diets such as the one used in this study (34). Conversely, HFD does not suppress TAG synthesis, thus TAG composition reflects re-esterification of diet-derived fatty acids (18, 33). As expected, we observed no diet-dependence on overall TAG concentration, however it was not possible to evaluate the second hypothesis as a lard-based diet is reflective of the endogenous fatty acids typical to most mammals. Additional work should be done to evaluate whether the down-regulation of DNL is of placental or hepatic origin.

The greater concentration of SM in the gestationally more developed placentae is likely a consequence of increased formation of lipid raft domains to process signaling and vesicle transport across the plasma membrane (35). Indeed, SM is critical for the function of TRP cation channels (36). In the fully developed placentae (D18.5) from TRPC1 -/- -NF dams the concentration of major SM species (SM 34:1 and SM 42:1) were lower than for WT-NF, due to a reduction in the presence of lipid raft domains coincident with the elimination of a TRP cation channel. SM species also act as substrate for initiation of sphingolipid signaling (35). Thus, the greater concentrations of SM 34:1 in TRPC1 -/- -HF placentae relative to WT-HF at both developmental periods suggests perturbation of sphingolipid homeostasis independent of gestational status. This is an important finding because Del Gaudio and colleagues noted accumulation of SM 34:1, SM 36:1 and SM 42:1 to the endothelium of feto-placental blood vessels from preeclamptic placentae, indicating a link between SM-accumulation and placental vascular development (37).

SM catabolism is the first step in a sphingolipid signaling cascade initiated by Toll-like receptor 4 (TLR4), a well-known pro-inflammatory mediator of innate immunity (16). The greater Cer concentration in HF-WT dams at both gestational periods (**Figure 2B**) mirrored the results observed by Holland et al. (16) in multiple tissues in response to an infusion of high-SFA lard oil. This is also in agreement with the pro-inflammatory role of diets high in SFA (38–40). The subsequent decrease in Cer concentration with increasing gestational age for both WT-HF and TRPC1 -/- -HF animals suggests a conserved role for Cer signaling in energetic homeostasis (14, 41). The increase in Cer species for the TRPC1 -/- -NF arm of the study may indicate in increased inflammatory load for these animals, though further characterization of inflammatory markers is needed to confirm this possibility.

TRPC1 mRNA and protein are expressed in human placentae (42) and upregulation of TRPC1 protein and subsequent increase in Ca^{2+} influx into the placental tissue may be a crucial step for decidualization (43). However, it is not yet known whether the absence of TRPC1 affects placental function *via* changes in placental lipid content, especially under maternal HFD. Data presented in our study showed placental lipid composition is modifiable due to absence of TRPC1 expression under maternal HFD, particularly at E12.5 of gestation. The decrease in cholesterol ester concentration for TRPC1 -/- animals (**Figures 3A, B**) with no concomitant genotype-dependent change in FC suggests either a decrease in CE formation or an increase in CE hydrolysis. Further investigation is needed to determine the role of enzymes that generate CE (lecithin cholesterol acyltransferase, sterol O-acyltransferase), or hydrolyze CE (cholesterol ester hydrolase). The increase in long-chain CE species for the TRPC1 -/- -HF-L arm indicates a possible compensatory mechanism for the formation of long-chain PUFA species.

For TRPC1 -/- animals we observed increased concentrations for TAG species with 56 or more acyl carbons and greater than 4 points of desaturation. In previous work it was observed that species in this regime contain long-chain PUFAs (18, 20). Diets were not supplemented with additional PUFA, which suggests this increase results from increased fatty acid elongation and TAG synthesis (33). We have observed PPARγ-dependent down regulation of oxidative metabolism in brown adipose from TRPC1-deficient mice (44) and reduced autophagy in adipose tissue (15), thus this may be an adaptation to sequester fatty acids as TAG species in lipid droplets to forestall the formation of a lipotoxic environment. Transcriptomic analysis for expression of sterol-regulatory element binding protein 1 (SREBP-1), sterol-CoA desaturase (SCD-1), diacylglycerol acyltransferases (DGAT1 and DGAT2), and ELOVL elongases would allow for the testing of this hypothesis. Unesterified fatty acids could also be assayed by fatty acid methyl ester analysis, though it would necessitate a larger sample collection that was available.

Our observation of TAG accumulation is congruent with observations from a study of functional complications in human placentae. Using infusion lipidomic methods like those employed in our study Brown et al. analyzed human placental biopsies from heathy pregnancies and pregnancies with complications due to preeclampsia or intrauterine growth restriction (45). Preeclampsia and intrauterine growth restriction were associated with greater TAG content, specifically in PUFA-containing species.

Lipidomic analysis presents an important window into energetic homeostasis, placental development, and inflammatory status, but complimentary techniques are needed to support some of the arguments above. The neutral lipid products quantified above implicate several enzymatic pathways for CE, TAG and fatty acid synthesis which could be addressed using transcriptomic analysis (46). Placental inflammation and vascular development could be assessed as we have in prior work (47). Limitations in sample availability preclude following up on these lines of inquiry for this study.

CONCLUSIONS

We performed an infusion based lipidomic analysis to determine the effects of gestational age, diet and elimination of the TRPC1 Ca^{2+} transport mechanism on the placental lipidome. Increasing gestational age resulted in increased unesterified cholesterol and sphingomyelin that may reflect increased plasma membrane fluidity and cross-membrane signaling. Changes in cholesterol ester and TAG content indicate the disruption of the TRPC1 Ca^{2+} may promote increased TAG storage.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by USDA Agricultural Research Service, Grand Forks Human Nutrition Research Center Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KC-L, JR, and BS designed the animal study. MB developed and performed the lipidomic analysis. The paper was prepared by MB and KC-L, with contributions by BS and JR. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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Corrigendum: Lipidomic Analysis of TRPC1 Ca^{2+}-Permeable Channel-Knock Out Mouse Demonstrates a Vital Role in Placental Tissue Sphingolipid and Triacylglycerol Homeostasis Under Maternal High-Fat Diet

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A Corrigendum on

Lipidomic Analysis of TRPC1 Ca^{2+}-Permeable Channel-Knock Out Mouse Demonstrates a Vital Role in Placental Tissue Sphingolipid and Triacylglycerol Homeostasis Under Maternal High-Fat Diet

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The original article contained an error. In the first sentence of the section on *Animal Protocol Design and Approval*, the control mouse strain was incorrectly identified as C57BL/6; the actual mouse strain used was B6129SF2/J. The sentence "Two-month-old female C57BL/6 mice (Envigo, Indianapolis, IN) were fed diets containing either 16% (normal-fat, NF) or 45% fat (high-fat, HF) for 12 weeks (**Table S1**)." should have read "Two-month-old female B6129SF2/J mice (Envigo, Indianapolis, IN) were fed diets containing either 16% (normal-fat, NF) or 45% fat (high-fat, HF) for 12 weeks (**Table S1**)."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Epigenetic Mechanisms Responsible for the Transgenerational Inheritance of Intrauterine Growth Restriction Phenotypes

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A poorly functioning placenta results in impaired exchanges of oxygen, nutrition, wastes and hormones between the mother and her fetus. This can lead to restriction of fetal growth. These growth restricted babies are at increased risk of developing chronic diseases, such as type-2 diabetes, hypertension, and kidney disease, later in life. Animal studies have shown that growth restricted phenotypes are sex-dependent and can be transmitted to subsequent generations through both the paternal and maternal lineages. Altered epigenetic mechanisms, specifically changes in DNA methylation, histone modifications, and non-coding RNAs that regulate expression of genes that are important for fetal development have been shown to be associated with the transmission pattern of growth restricted phenotypes. This review will discuss the subsequent health outcomes in the offspring after growth restriction and the transmission patterns of these diseases. Evidence of altered epigenetic mechanisms in association with fetal growth restriction will also be reviewed.

Keywords: intrauterine growth restriction, uteroplacental insufficiency, small for gestational age, transgenerational transmission, epigenetic mechanisms, cardiometabolic disease, kidney dysfunction

INTRODUCTION

Intrauterine growth restriction (IUGR) refers to poor growth during pregnancy, which results in babies being born small for gestational age (SGA), and with low birth weight (LBW) (1). One of the common causes of IUGR is uteroplacental insufficiency (UPI), in which the placenta functions poorly, causing an insufficient supply of oxygen and nutrients to the developing fetus (2).

There is a high prevalence of IUGR worldwide, especially in developing countries [approximately 27% of all live births (3)], which is a significant concern, as epidemiological studies have shown that being growth restricted is associated with an increased risk of developing chronic diseases later in life (1, 4–9). In addition, various animal models have shown that IUGR offspring develop kidney dysfunction and cardiometabolic disease later in life (2, 10–15).

Interestingly, these IUGR phenotypes are sex-specific and their transmission is multigenerational through both the maternal and paternal lines (11–14, 16–18).

The underlying mechanisms of how IUGR predispose offspring to chronic disease later in life remains to be determined. However, epigenetic mechanisms may be involved as they have been shown in several animal studies to be potential mechanisms for the multigenerational transmission of disease (17).

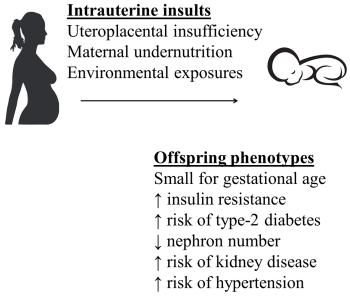
INTRAUTERINE GROWTH RESTRICTION AND CHRONIC DISEASE RISK

Hypertension and Kidney Disease

Epidemiological studies in humans have reported that growth restricted infants have an increased risk of developing chronic diseases later in life [**Figure 1** (5–8, 19–24)]. For instance, IUGR children at 6 years of age have been shown to have a 1.8 times higher risk of developing hypertension compared to non-IUGR children (6). Additionally, individuals born SGA had increased systolic and diastolic blood pressure by 4.5 and 3.4 mmHg, respectively, at the age of 50 (5). When these results were adjusted for confounding factors such as sex, age, and body-mass index, IUGR was still significantly associated with hypertension (5, 6). In other studies, when sex is taken into consideration, the development of hypertension in association with LBW can produce conflicting results. For example, there was one study that found an association in IUGR males only (24), while a different one found an association only with IUGR females (23). However, differences in the size of the study (15600 vs 976 children), method of measuring blood pressure (one-time systolic and diastolic blood pressure measurement vs 24h systolic blood pressure measurement), and the age of examined children [3-6 years old (24) vs 6-16 years old (23)] may be factors that contributed to the observed sex-specific differences. In line with this finding, an inverse relationship was found between birthweight and blood pressure of IUGR infants in a study that examined 1310 junior high school students (20). However, this relationship was then lost as the children reached adolescence (12-14 years of age), even when adjusted for confounding factors. This suggests that there might be a possible adaptation mechanism in the adolescents to overcome IUGR-related hypertension.

Unlike the examination of hypertension by measuring blood pressure, the precise determination of kidney disease mostly requires more invasive measurement methods, such as counting of glomerular number after organ collection and sample sectioning (7, 8). Therefore, few studies are carried out in humans, especially in growth restricted infants, to evaluate the association between IUGR and kidney disease. However, papers published by Wang et al. in 2014 (9) and 2016 (41), respectively, were two of the rare studies that investigated the effect of human aberrant fetal growth environment on kidneys of fetuses. In both studies, fetuses and their kidney samples were collected from mothers who terminate their pregnancy due to preeclampsia (9), placental abruption, deformities of fetuses, and other intrauterine insults (41). Both papers reported negative effects that IUGR had on the fetuses, including significantly low birth weight (< 2 kg), approximately 0.4 times less nephron number, increased expression of pre-apoptosis proteins within kidney tissues (9), and reduced renal renin-angiotensinogen RNA levels by half the non-growth restricted fetuses (41). This is significant, as the renin-angiotensinogen system is known to play a crucial role in maintaining the sodium homeostasis within the kidney, as well as regulating blood pressure, especially during pregnancy (41). These papers are consistent with studies that have shown a decline in glomerular number (more than 20%) in low birth weight individuals who died from cardiovascular disease as adults, in comparison to normotensive people (7, 8). Together, these studies suggest an important contribution of the kidney to hypertension development in IUGR individuals.

Using different animal models, the association between IUGR and the development of chronic diseases can also be evaluated [**Figure 2** (2, 10–15, 18, 42–57)]. In the early 2000s, the association between IUGR induced by UPI and blood pressure level was studied using a model in which placental insufficiency



Tissue	Epigenetic change
Fetal blood	DNA methylation: ↓ *IGF2* DMR
Umbilical cord	↑ *miR-576-5p* ↑ *HNF4A* DNA methylation ↓ mitochondrial DNA methylation
Placenta	lncRNA expression changes: ↓ *MEG*, ↑ *H19*, *NEAT1*, *lnc-PPM1D-1*, *lnc-TCL1B-1*, *lnc-MRPS5-1*, *lncTRPM7-1*, *MED4-AS1*, *EGFR-AS1*, *FLJ31356*, *lnc-VAPA-1* and *STON1-GTF2A1L*
	Altered miRNAs: *miR-499a-5p*, *miR-26a-5p*, *miR-103a-3p*, *miR-145-5p, miR-193b-5p*, *miR-210-5p*, *hsa-miR-210-3p, hsa-miR-193b-3p, hsa-miR-5p, hsa-miR-365a-3p, hsa-miR-365b-3p, hsa-miR-520a-3p*
	DNA methylation: ↓ *H19* ICR, altered *RLT1, AIM1* and *N4BP2L1* DNA methylation, ↑ *MCTS2* DMR methylation, ↓ *SNU13* DMR methylation

FIGURE 1 | Small for gestational age (SGA) babies who were exposed to intrauterine insults have altered epigenetic mechanisms and aberrant physiological changes, predisposing them to an increased risk of developing various chronic diseases later in life (5–8, 19–40).

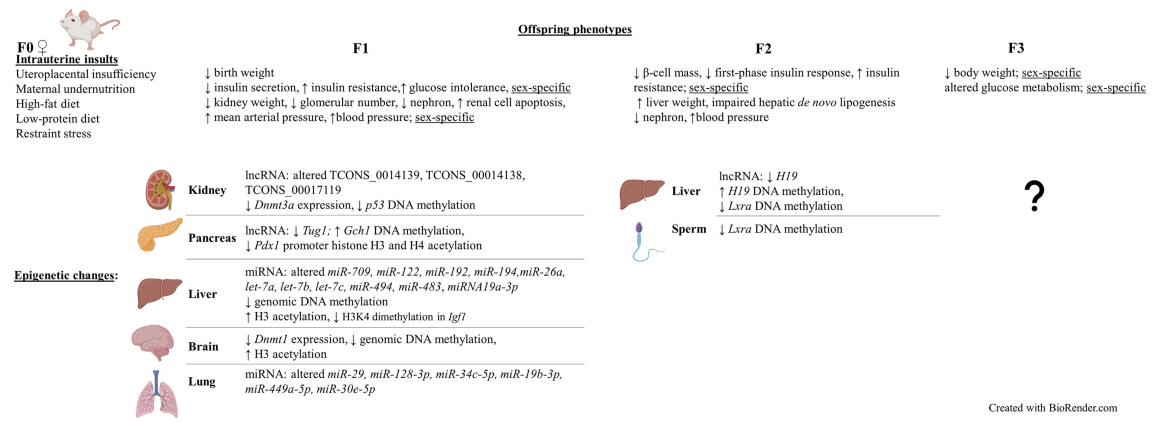


FIGURE 2 | Intrauterine growth restriction (IUGR) phenotypes are sex-specific and can be transmitted to subsequent generations, including the restricted F1 and non-restricted F2 and F3 offspring (intergenerational). Similar to human studies, altered epigenetic mechanisms such as non-coding RNA modifications, DNA methylation, and histone modifications were also found in these offspring. Results obtained from differerent rat and mouse IUGR models (2, 10–15, 18, 42–70).

was established by placing silver clips around the abdominal aorta and on the branches of uterine arteries of pregnant rats at day 14 of gestation, which severely reduced blood flow between mother and the fetus (43). UPI-induced rats produced LBW offspring, 12% lighter in weight compared to control, with an increased risk of developing hypertension in both IUGR males and females, as their mean arterial pressure at 8 weeks of age was 12 mmHg higher than the control (43). However, at 12 weeks of age, only the increased mean arterial pressure in F1 male offspring was still significant, suggesting a sex-specific hypertension maintenance mechanism. There was no statistically significant association between the observed increased arterial pressure and renal function of the offspring found in this study. Glomerular filtration rate, effective renal plasma flow and 24-hour sodium excretion were not different in IUGR rats compared to the control, even when they were adjusted for kidney weight (43). Meanwhile, the bilateral uterine vessel ligation model produced restricted F1 male offspring that had higher blood pressure and an enlargement of the heart's left ventricle at 22 weeks of age, compared to the control, as a consequence of persisting high blood pressure (10). Lower body weight and glomerular number (clusters of capillaries in the kidney, reduced by 27% of the control) were also reported at 6 months of age (10). These results were reproducible in other studies, with lower kidney weight (measured at postnatal day 1 and 7) and nephron deficit (at 18 months of age) occurring in both sexes and hypertension (at 18 months of age) being present only in male rats (2, 11, 53). Glomerular hypertrophy, an outcome to compensate for the IUGR-related glomerulus reduction, was found to be higher in the F1 growth restricted male rats compared to females at day 120 after birth, suggesting a sex-specific response of the growth restricted offspring towards kidney injury (15). Similarly, 18 month old growth restricted female rats had preserved mesenteric and renal arterial smooth muscle and endothelial function, which may in part explain why they did not develop hypertension (48). However, the mechanisms behind this remains to be identified. Interestingly, the transmission of hypertension and kidney diseases is multigenerational, as reduced nephron number, left ventricular hypertrophy and hypertension were reported in the non-restricted F2 generation (13, 14).

Apart from rats, studies of UPI-induced IUGR in other animal models [e.g. rabbit (71) and guinea pig (72)] also support the association between IUGR and reduced glomerular number and/or hypertension in the growth restricted offspring. IUGR induced by other intrauterine causes was also shown to be associated with hypertension or aberrant renal function and development (50, 52, 56). For example, F0 pregnant rats were fed a 50% deficit food intake diet throughout pregnancy to produce growth restricted F1 offspring (56). F1 males were mated with control healthy females to produce the F2 generation (paternal line). In a normoxia environment (where the oxygen concentration is normal), mean pulmonary arterial pressure, right ventricular hypertrophy index, and media wall area thickness were not significantly different between IUGR and the control males, in both generations. However, F1 and F2 male rats that were placed in an oxygen-deficient chamber for 2 weeks showed an increase in all three mentioned parameters, indicating an increased risk of developing pulmonary arterial hypertension later in life (56). In line with this finding, the expression of endothelin-1 (ET-1), a vasoconstrictor that is important for cell proliferation, cell migration, and blood vessel development, was significantly increased in pulmonary vascular endothelial cells (PVECs) extracted from F1 and F2 IUGR males. This led to aberrant PVEC proliferation, migration, and angiogenesis, all of which are signs of pulmonary vascular endothelial dysfunction (56). On the other hand, in 6-month-old LBW restricted rats whose mothers received only 50% the calories during pregnancy, there was a significant reduction in kidney weight (maximal value only reached 91% of the control) and glomerular number (by 27% the control) (52). Meanwhile, a low-protein diet

(reduced by 11% of the control) in pregnant rats resulted in a significant decrease in glomerular number (by 22.6% the control; at 3 months of age) and increased renal cell apoptosis of LBW F1 offspring (50).

Diabetes

Besides hypertension and kidney dysfunction, diabetes is another disease that has been shown to be associated with IUGR. Women whose birth weights were less than 2.5 kg [typically the clinical definition for LBW (73)] have a 1.83 times higher risk of developing type 2 diabetes as they age compared to women with birthweights above this threshold (19, 21). Decreased insulin-stimulated glucose uptake, or insulin resistance, one of the common hallmarks of type 2 diabetes, was also reported in IUGR young adults whose birth weights were below the 10th percentile for their gestational age (22).

Different animal models can be used to study the association between IUGR and the development of type-2 diabetes, such as UPI model that has metabolic characteristics comparable to that of humans (18, 42, 45), IUGR rats induced by maternal calorie restriction (57, 74), or IUGR fetal sheep induced by exposing pregnant ewes to an environment with highly increased humidity and temperature (75). When both uterine arteries of pregnant rats are ligated at day 19 of gestation to imitate UPI occurring in pregnancy, F1 growth restricted rat offspring had significantly lower birth weight (5.96 g) compared to the sham control offspring (7.00 g) (42). Rat offspring in both restricted and control group then reached relatively similar body weights at approximately 7 weeks of age. However, as the IUGR F1 rats aged, they had significantly reduced insulin secretion of β-cells (by half the control at 1 week of age and completely absent at 26 weeks of age), insulin-resistance and glucose-intolerance hence hyperglycemia (42). Similar findings were also reported in other studies that applied the same UPI-inducing method of uterine arteries ligation (18, 45). Three months old growth restricted F1 rats developed hepatic insulin resistance, which was represented by its impaired insulin function in controlling the hepatic glucose production (HGP) important for maintaining blood glucose equilibrium (1.6 times higher HGP in IUGR rats compared to the control) (45). A decrease by 50% of pancreatic insulin content was also reported in the LBW growth restricted rats compared to control at the same time point of age (18). Moreover, there was a sex-specific reduction of β-cell mass in these restricted offspring compared to the control, with 40% and 50% reduction in IUGR males and females, respectively (18).

Similar to the observations for hypertension and kidney disease risks in IUGR animal studies, both the F1 and F2 generations are at a higher risk of developing diabetes, suggesting that there is a multigenerational transmission of IUGR phenotypes (12). When growth restricted F1 female rats were mated with healthy males, 6-month-old F2 offspring also had altered pancreatic β-cell mass (reduced by 29% in males and increased by two-fold the control in females) and first-phase insulin response (reduced by 35% in males and 38% in females) (12). The sex-specific differences in pancreatic β-cell mass between 3 months old F1 rats (18) and 6 months old F2 rats might be due to the difference in time point in which they were examined. For instance, at 6 months of age, female rats may have developed compensatory mechanisms for the disease. Additionally, as these defects were resolved when the rats aged [determined at 12 months of age (12)], male rats may have also developed similar mechanisms at a later age. However, this remains to be shown. In a different IUGR model where F0 pregnant rats were injected with the corticosteroid dexamethasone, from day 15 to 21 of gestation, F2 offspring had reduced birth weight and F2 5-week-old males developed glucose tolerance, represented by a significant increase in the activity of hepatic phosphoenolpyruvate carboxykinase (PEPCK), an enzyme that is involved in glucose metabolism (58). Additionally, these F2 growth restricted males were reported to have higher plasma glucose level at 4 months of age, and higher basal insulin level at 6 months of age, compared to the control (58). Similarly, when F0 pregnant rats received a restricted diet (food intake reduced by 50% the control) from day 11 to 21 of pregnancy, the effect of IUGR on insulin resistance was also seen in a multigenerational pattern (57). Specifically, F1 restricted females were also given a restricted diet from day 1 to day 21 postnatal. At 2 months of age, F1 females were mated with control males, and F2 1-day-old embryos were transferred to control recipient females. F2 female offspring from the IUGR group had significantly higher liver weight, baseline fasting plasma glucose, and insulin concentrations, despite a similar weight from birth to 15 months postnatal, compared to the control (57). The F2 IUGR group also developed insulin resistance at 15 months of age, represented by reduced plasma glucose/insulin ratio during glucose tolerant test, and lower concentration of plasma membrane-associated GLUT4, a protein that plays an important role in insulin-dependent glucose transport into skeletal muscles. Reduced function of PKCζ, an enzyme involved in insulin-signalling pathway, was also found in skeletal muscle of 15 month old F2 females in the IUGR group (57). Likewise, in a model of *in utero* low-protein consumption in rats, there was an adverse effect of IUGR on the glucose metabolism of F3 offspring at approximately 2 months of age (46). To be specific, there was a significantly higher fasting plasma glucose level in F3 females compared to sham. Meanwhile, there was a significant increase in insulin level of F3 males compared to sham at both fasting stage and 30 minutes after the glucose injection (46), suggesting that IUGR phenotypes are sex-specific and their transmission can be intergenerational. In line with this finding, reduced body weight at days 1 and 7 after birth by 0.5 g, compared to the control offspring, was also reported in the F3 rats whose grandmothers were exposed to restraint stress during pregnancy (55). Additionally, these animals were reported to have sensorimotor dysfunction at postnatal day 7, as their response time during the inclined plane test was significantly slower compared to the control (55). On the other hand, altered glucose tolerance and insulin secretion could be improved (determined in F1 male rats at 6 months of age) by cross-fostering the UPI-induced growth restricted offspring to a sham control mother for lactation, a period important for offspring development (47). This proposed that there could be reversal

strategies for IUGR-related diseases and/or solution to modify their effects on the growth restricted offspring. However, intervention studies are beyond the scope of this review. Additionally, it should be noted that when IUGR was caused by a severe maternal protein-restriction diet (e.g. 5 g of protein/100 g of diet) during pregnancy, postnatal catch-up could be impaired (59). Male and female rats at 6 months of age had significantly increased fasting serum glucose level (20% and 25% the control values in F1 and F2 generation, respectively), despite being fed a control diet during lactation (59). F1 and F2 male offspring also developed insulin resistance at 6 months of age (59).

In summary, the above observations of IUGR infants having an increased risk of developing various diseases later in life are in line with the Developmental Origins of Health and Disease hypothesis, proposing that adverse events that occur during the maturation of gametes, at conception and early embryonic development can program long-term risks of chronic diseases in the LBW offspring (76). As the world-wide prevalence of type 2 diabetes, chronic kidney disease and hypertension is significantly high [6.28% for diabetes, 9.1% for kidney disease, and approximately 30% for hypertension (in adults) (77–79)], there is an urgency for researchers to investigate IUGR and its mechanisms in programming chronic diseases in humans. Nevertheless, due to the complexity and ethical rules in human research, most in-depth experiments that study IUGR are carried out in rodents. Additionally, animal models provide a mechanism for investigating the impact of IUGR across multiple generations and to determine the possible molecular mechanisms involved.

INTRAUTERINE GROWTH RESTRICTION AND THE ASSOCIATED ALTERED EPIGENETIC MECHANISMS

Epigenetic Mechanisms

Epigenetics can be described as heritable modifications to the chromatin that regulate gene expression without altering the DNA nucleotide sequence (80). An example of a modification that creates such changes is DNA methylation. DNA methylation involves the DNA methyltransferase-catalysed addition of a methyl group to a DNA cytosine base (81). In mammals, DNA methylation happens primarily at CpG sites, that is, a cytosine adjacent to a guanine base in the 5'-3' direction. DNA methylation at gene regulatory regions such as promoters is associated with gene silencing (82). Additionally, DNA methylation has an important role in other processes such as X-chromosome inactivation and imprinting (83).

Another epigenetic modification is histone modifications. These include histone acetylation, which is the addition of an acetyl group to the lysine residue of the nucleosomal core histones' N-terminal tail (81). Histone deacetylation, specifically at histones H3 and H4 is associated with gene repression (84). In addition to this, regulatory non-coding RNAs are known to be involved in transcriptional, post-transcriptional, and translational regulation hence are also involved in gene regulation (85, 86). Two large subsets of non-coding RNAs are long non-coding RNAs which are > 200 nucleotide-long, and short non-coding RNAs like microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) which are all less than 200 nucleotide-long (87). Altered expression of both miRNAs and lncRNAs have been shown to be associated with altered histone modifications and DNA methylation status of genes (88, 89).

Studies of Blood Samples

IUGR has been shown to be associated with altered epigenetic mechanisms [**Figure 1** (25–38)].

Blood samples from the Dutch Hunger Winter famine were used to investigate DNA methylation from individuals exposed to reduced calorie intake in very early (60 people) or late (62 people) gestation (90). Although there was no significant difference in birth weight among the individuals (26), there was a decrease by 5.2% in DNA methylation of the *IGF2* imprinted gene differentially methylated region (DMR) in people exposed to famine in early gestation, compared to the same-sex siblings who were not exposed to famine during pregnancy. Whilst, people exposed to famine in late gestation had no altered DNA methylation (90). This observation suggests the importance of the timing of exposure to intrauterine insults, specifically during the early developmental stage, in which epigenetic mechanisms within the fetus is programmed and may be permanently maintained into adulthood. In a different study, blood samples from 24 IUGR infants were investigated using Illumina Human Methylation 450 k array to analyse differences in genome-wide DNA methylation and gene expression, compared to data from 12 control healthy infants (60). Within the IUGR group, 5460 differentially methylated CpG loci from 2254 genes were identified. Using Kyoto Encyclopedia of Genes and Genomes database, more than 50 pathways affected by changes in the methylation status of these gene were determined, such as metabolic pathways, antigen processing and presentation, apoptosis, insulin signalling pathway, and neurological disorder pathways (60). In addition to this, increased DNA methylation, by 6.1% the control, of the type 2 diabetes-related *HNF4A* gene promoter was found in CD34+ stem cells from umbilical cord blood samples of IUGR newborns (27). More than 800 genome differentially methylated positions (DMPs) was found in leucocytes from umbilical cord blood samples of IUGR neonates, compared to the control (91). These DMPs were located within genes that are critical for key cellular processes that impact the fetal growth and development, such as organogenesis, metabolism, and immunity. *D-loop* hypomethylation of mitochondrial DNA found in fetal cord blood samples was also reported in IUGR neonates who were exposed to placental insufficiency (32). The hypomethylation was in association with higher mitochondrial biogenesis (i.e. increased mitochondrial DNA levels), which is a possible mechanism to compensate for reduced oxygen by UPI. Results in these studies were all adjusted for other complications that may have occurred during pregnancy such as gestational hypertension, gestational diabetes and preeclampsia (27, 32, 91).

Besides DNA methylation, altered expression of miRNAs have been recently reported in human umbilical cord tissues

collected from IUGR pregnancies (35). To be specific, the study included samples from IUGR children with or without growth catch-up at 1 and 6 years of age, and control children who were born appropriate for gestational age (35). At 1 year of age, the expression of a miRNA *miR-576-5p*, which is known to be involved in kidney and liver diseases, was significantly enhanced in IUGR with catch-up children compared to both IUGR without catch-up and control children (35). Moreover, within the IUGR with catch-up group, *miR-576-5p* expression was shown to have a significant association with weight, height, catch-up weight, and catch-up height of the children, after being adjusted for confounding factors such as sex, gestational age, maternal smoking status, etc. Besides the mentioned parameters, at 6 years of age, *miR-576-5p* expression was also shown to be associated with renal fat, suggesting an important role of *miR-576-5p* in cardiometabolic diseases, and that alterations of this miRNA due to IUGR may increase the risk of developing these diseases later in life (35).

Studies of Kidney Tissues

Animal studies that specifically focused on altered epigenetic mechanisms in the UPI-induced IUGR offspring have also been carried out in different organs and tissues [**Figure 2** (44, 49, 51, 53, 54, 61–68)]. Decreased expression, by 19% the control, of *Dnmt3a*, a gene that is responsible for *de novo* DNA methylation was found in kidney tissues of F1 IUGR rats at embryonic day 20 (53). Meanwhile, decreased apoptosis-suppressing *Bcl-2* gene expression and increased pro-apoptotic protein-encoding *Bax* and *p53* expression were identified in kidneys of F1 IUGR rats at term, which was associated with reduced glomerular number (by 23% the control) of rat pups (44). Correlatively, there was reduced DNA methylation of CpG islands at the promoter region (by 56.3% the control) of *p53* (44).

Whilst, significantly altered expression of three long non-coding RNAs (lncRNAs) (TCONS_0014139, TCONS_00014138, and TCONS_00017119) at day 1 and day 10 postpartum (pn1 and pn10), confirmed by both microarray and qPCR, were found in kidneys of LBW male rats whose mothers were also fed a low-protein diet during pregnancy (51). The altered expression of these lncRNAs were associated with altered mRNA expression at pn1 and pn10 of *MAPK4*, which encodes for a protein that involves in renal ureteric bud morphogenesis. Additionally, the aberrant expression of these lncRNAs is also correlated with a decrease in nephron number of LBW rats at pn1, suggesting an important role of them in nephron endowment (51). Furthermore, altered expression of *Cdkn1c* and *Kcnq1*, two imprinted genes that are regulated by the *Kcnq1ot1* lncRNA, was found in kidney tissues of UPI-induced IUGR rats at day 1 after birth (53). However, further research is required to investigate whether changes to *Kcnq1ot1* was the epigenetic mechanism that affected the imprinted gene expression in this study.

Studies of Liver Tissues and Pancreas Tissues

Similar to results obtained from blood samples and kidney tissues, abnormal DNA methylation have also been found in hepatic tissues from IUGR studies (49, 65, 66). Importantly, in hepatic tissues, the multigenerational transmission and reversibility of the altered epigenetics was detected in F2 non-restricted offspring (66). Growth restricted F1 rats that underwent intrauterine UPI were fed with either a control diet or essential nutrient supplemented (ENS) diet (i.e. rich in methyl donors) and bred spontaneously to produce the F2 offspring (66). Within the F2 generation, 21-day-old rats whose F1 mothers received a control diet had statistically reduced DNA methylation of the *H19* gene promoter (7% less than the sham lineage), in association with reduced *H19* expression (0.4-fold the sham lineage) (66). Meanwhile, 21-day-old F2 rats whose F1 mothers received ENS diet had increased *H19* promoter methylation (34% more than the sham lineage), with a 6.6-fold increase in *H19* expression (66). In line with this finding, F2 offspring of pregnant mice that were fed with only 50% the control group's food intake had significantly lowered expression of the *Lxra* gene ($p < 0.01$) in their liver tissues, which plays a key role in *de novo* lipogenesis (49). Hepatic *de novo* lipogenesis was also impaired in the F2 adult mice (49). Furthermore, this was associated with statistically reduced methylation within the 5'UTR region of *Lxra*, both in the sperm samples of IUGR F1 males and liver samples of non-restricted F2 fetuses and adult mice. Therefore, it is suggested that there was a multigenerational transmission of altered *Lxra* methylation within both F1 and F2 generations (49). Meanwhile, one of the first studies to investigate whole-genome DNA methylation from pancreatic islet samples in the UPI-induced IUGR 7-week-old male rats discovered 1912 differentially methylated loci compared to the control, most of which occurred within the non-coding intergenic sequences between genes rather than promoter regions (65). Interestingly, the differential methylation was 45kb upstream of genes known to be important for homeostasis-maintaining processes (e.g. *Fgrf1*, *Gch1*, and *Vgf*) and were correlated with altered expression of these genes (65).

UPI-induced IUGR in several animal studies has also been shown to be associated with altered histone modifications (61–63, 69, 92). Histone H3 hyperacetylation, increased to 233% the control value, was detected in the liver of UPI-induced IUGR newborn rats, in association with hepatic genomic DNA hypomethylation (reduced methylation by 13.7% the control at day 21 after birth) (61). On the other hand, significantly reduced dimethylation at H3K4 in the *Igf1* region was reported in livers of IUGR rats whose mothers had a food restriction during pregnancy (69). Meanwhile, locus-specific assessment of the *Pdx1* gene, a gene important for β-cell development and function, showed loss of *Pdx1* promoter H3 and H4 acetylation at 6 months of age and significant DNA hypermethylation (increased by 51.3% the control) in the pancreatic islets of F1 IUGR adult rats, and was associated with silencing of *Pdx1* (mRNA level reduced by 50.4%) (63). This may contribute to the later onset of type-2 diabetes in the growth restricted offspring.

In regards to non-coding RNAs, not many IUGR studies have been carried out to investigate their changes in the growth restricted offspring. Nonetheless, in agreement with results obtained from the placentas in human studies, reduced expression of the lncRNA *H19* and reduced DNA methylation status of its promoter region were reported in hepatic tissues of F2 growth restricted rats whose grandmothers (F0) underwent

UPI (66). In hepatic tissues of F1 growth restricted mice whose mothers were fed with a high-fat diet pre-, during and post-pregnancy, there was also a significant reduction in expression of the miRNAs, including *miR-709, miR-122, miR-192, miR-194, miR-26a, let-7a, let-7b, let-7c, miR-494* and *miR-483* (64). Interestingly, a major of the altered miRNAs are predicted to have a common target, which is methyl-CpG binding protein 2 (64). In a different study where F0 pregnant mice were fed a low-protein/calorie-deficit (-40%) diet from week 3 of gestation, and growth restricted pups were cross-fostered to 3 different groups right after birth, either normal milk feeding (6 pups/dam), overfed (3 pups/dam), or nutrition restriction (10 pups/dam), significantly reduced H3K4me3 (trimethylated histone H3 on lysine 4) region at the *Akt1* gene, a gene that is known to play an important role in insulin resistance, was found in livers of 3-month-old IUGR males that either received normal milk feeding or were overfed, in association with reduced expression of *Akt1* (93). Interestingly, higher protein level of PTEN, one of the *Akt* activation inhibitors, was also found in livers of overfed 3-month-old males. In addition to this, significantly decreased levels of circulating *miRNA19a-3p*, a miRNA that acts to regulate PTEN, were found in both normally fed and overfed IUGR males (93). These finding hence suggests an association between altered epigenetic mechanisms and the risk of developing insulin resistance later in life of IUGR offspring. Indeed, compared to F1 healthy control males, males that were either under nutrition restriction or overfed both had an increase in sensitivity to insulin at 3 months of age (93). At 12 months of age, the sensitivity to insulin increased for the nutrition restriction group but attenuated for the overfed group. Meanwhile, IUGR males that received normal milk feeding showed no difference in insulin sensitivity compared to healthy control males at 3 months of age. However, at 12 months of age, they developed insulin resistance (93). On the other hand, in pancreatic islets of growth restricted mice whose mothers were fed a low-protein diet, the expression of *Tug1*, a lncRNA that involves in diabetes and tumour development, were significantly lower at 1 day, 8 weeks, and 12 weeks post-partum, compared to the control (54). The aberrant glucose tolerance observed at 10 weeks old IUGR mice could be partially rescued by injection of 150 μg of *Tug1* overexpression sequence, suggesting that *Tug1* may play an important role in the mouse pancreatic development and function (54).

Studies of Placental Tissues

Altered DNA methylation of genes that are important for fetal growth and development has been reported in placentas from both human and animal IUGR pregnancies (34, 39, 70, 94). For example, placenta samples from healthy and complicated human pregnancies were investigated using the Illumina Infinium Human Methylation450 BeadChip arrays (HM450k) array platform (67 samples) and quantitative pyrosequencing (127 samples) (39). Specifically, 35 DMRs that are expressed across tissues (ubiquitous) were identified. In general, DNA methylation status of all DMRs was not significantly different between complicated pregnancies and the control group. However, DNA hypermethylation was found at the *MCTS2* DMR, while hypomethylation was found at the *SNU13* and *H19* ICR in IUGR placentas (39). Additionally, RT-PCR and Sanger sequencing confirmed that *H19* hypomethylation results in the biallelic expression of *H19* in the IUGR group. Similarly, a loss of methylation in *SNU13* is associated with increased expression of this gene in the IUGR placentas. Interestingly, despite a similar DNA methylation status compared to that of the control, there was an increase in expression of *ZNF331* and a decrease in expression of *PEG10* and *ZDBF2* in the IUGR placentas (39). For DMRs that are placenta-specific, the same HM450k array data was used, and results were also confirmed using pyrosequencing. Out of 32 placenta-specific DMRs, methylation status of *AIM1* and *N4BP2L1* was significantly different in the IUGR group compared to the control. However, using microfluidic-based quantitative RT-PCR analysis, only four placenta-specific genes that had altered expression in IUGR samples compared to the control were identified, all of which were reduced in IUGR, including *ADAM23*, *GPR1-AS1, LIN28B*, and *ZHX3* (39). In line with this, altered DNA methylation of CpG island 1 of *RLT1*, a gene known to be important in placental development, was found in placenta samples from SGA and severe SGA foetuses, compared to healthy controls (34). Whilst, genome-wide DNA methylation patterns were investigated in placentas of IUGR identical twins who shared the same placenta (monochorionic twines) and had significant growth difference, represented by birthweight variations in the range of 21-59% (94). In placental tissues of IUGR twins, altered DNA methylation status (with differences larger than 10% compared to healthy control twins) were identified in DMRs that overlapped the promoters of 8 genes that are known to be important for lipid metabolism and neural development, including *DECR1, ZNF300, DNAJA4, CCL28, LEPR, HSPA1A/L, GSTO1*, and *GNE* (94). These results were still significant after being adjusted for twins' sex, gestational age, and maternal age. Interestingly, *DECR1* and *GSTO1*, the two genes play a role in fetal growth, have also been altered in other IUGR studies in animal and human singleton pregnancies, suggesting potential shared molecular mechanisms in comparison to the IUGR growth-discordant monochorionic twins (94). Meanwhile, at embryonic day 10.5, altered DNA methylation was found within 20 different DMRs of imprinted loci from the placentas of F2 mice, whose grandmothers had a hypomorphic mutation in methionine synthase reductase (*Mtrr*), a gene that is important for methyl group utilisation and maternal folate metabolism (70). Changes in DNA methylation status of these DMRs were associated with changes in expression of imprinted genes such as *Zdbf2*, *Igf2*, and *Dlk1*, all of which play a key role in fetal development (70). As expected, growth restriction, delayed development, and defects of different organs including brain, heart and placenta were also found in these offspring (70), suggesting a multigenerational transmission of IUGR phenotypes and altered epigenetic mechanisms.

Altered expression of long non-coding RNAs that are important for angiogenesis, inflammation fetal growth has also

been reported in placentas collected from pregnancies that are affected by IUGR (25, 28, 31, 38, 40). The investigation of 30 IUGR and 46 gestational age-matched non-IUGR placentas revealed decreased expression of *MEG3*, a lncRNA that is involved in placental and fetal growth, by more than 50% in the IUGR samples compared to the non-IUGR control (25). In line with this, the expression of *H19*, another lncRNA that is important for fetal development, was also reduced by half the non-IUGR control in IUGR placentas (31). This reduction in *H19* expression was shown to be strongly correlated with a 50% decrease of expression of the type III TGF-β receptor (TβR3), one of the downstream signalling molecules that control trophoblast cell migration and invasion (31). However, in a different study, the expression of *H19* was shown to be similar between IUGR and non-IUGR placental tissues (28). Nonetheless, in IUGR placentas, there was a significantly lower DNA methylation status in the imprinting control region 1 (ICR1), which regulates the expression of *H19*, in comparison to the control (28). Increased expression of another lncRNA *NEAT1*, a gene expression regulator which expression in usually up-regulated in human cancers, was also seen in placentas from IUGR pregnancies with a 4.14-fold increase in IUGR placentas compared to the control (29). In contrasts, in a different study, *NEAT1* expression in the placentas was not statistically different between IUGR and the non-IUGR group (95). Differences in sample size, ethnicity, or maternal age might be an explanation for the differences in *H19* and *NEAT1* expression in IUGR placentas in the above studies. In a recent study, altered expression of 133 lncRNAs (36 increased in expression and 98 decreased in expression, in comparison to non-IUGR control) was reported in placentas from 12 pregnant women whose pregnancies were complicated with IUGR (38). Interestingly, the overexpression of several lncRNAs such as *lnc-PPM1D-1*, *lnc-TCL1B-1*, *lnc-MRPS5-1*, *lncTRPM7-1*, *MED4-AS1*, *EGFR-AS1*, *FLJ31356*, *lnc-VAPA-1* and *STON1-GTF2A1L* in the IUGR group was also found in placentas from the pregnancy group affected by preeclampsia (38). Most of these lncRNAs have been shown to play a role in pathways that lead to placental ischemia, which results in reduced blood supply to the placenta (38). This suggests that these pregnancy complications might act *via* some shared mechanisms and/or there are similar signalling pathways can be activated by them.

Similar to the observations for lncRNAs, there are miRNAs that have been shown to be altered in placentas from both IUGR and preeclampsia pregnancies, such as *miR-499a-5p*, *miR-26a-5p*, *miR-103a-3p*, *miR-145-5p* (30), *miR-193b-5p* (36), *miR-210-5p* (37), *hsa-miR-210-3p, hsa-miR-193b-3p, hsa-miR-5p, hsa-miR-365a-3p, hsa-miR-365b-3p*, and *hsa-miR-520a-3p* (33), most of which play a role in cellular functions, including cellular differentiation, migration and invasion, suggesting shared signalling pathways and mechanisms between these pregnancy complications.

Studies of Other Tissues

The focus of this review is on the risk of developing renal and cardiometabolic diseases, such as hypertension and diabetes, in association with IUGR induced by UPI. Therefore, most of the studies reported are on either blood samples, kidneys, livers, pancreas, or placentas. However, it should be noted that there are other tissues that can also be affected by IUGR, such as lungs or brains. For example, when F0 pregnant rats were fed a 50% deficit food intake diet throughout pregnancy, in PVECs extracted from the F1 and F2 IUGR rats, there was a significant enrichment of H3K4me3 regions in F1 IUGR males, and a significant reduction in DNA methylation at ET-1 CpG sites in both F1 and F2 IUGR males, compared to the control (56). Interestingly, ET-1 CpG methylation was also significantly reduced in F1 IUGR rat sperm, suggesting epigenetic modifications as a potential mechanism for the multigenerational transmission of these IUGR phenotypes, *via* the paternal line (56). In line with this finding, altered expression of various miRNAs were found in lung tissues at day 10, day 21, and 5 months after birth of IUGR rat offspring whose mothers were either undernourished (68) or fed with a low-protein diet (67) during pregnancy. Most of these miRNAs (*miR-29, miR-128-3p, miR-34c-5p, miR-19b-3p, miR-449a-5p*, and *miR-30e-5p*) are involved in lung development and injury-repair (67, 68). Microarray analysis and homologous analysis of brain tissues containing hippocampus from growth restricted F1 rats whose mothers received a 50% reduction in food intake throughout pregnancy also revealed 49 rat genes that are homologous in humans, and had a negative correlation between gene expression and DNA methylation status (60). Most of these genes are involved in metabolism pathways, nervous system dysfunction, cancer, and immune response regulation (60). Increased cerebral total H3 acetylation (to 157% of control values), decreased genome-wide DNA methylation (to 52.8% the control), and decreased CpG island methylation (to 65.0% the control) were also found in brains of IUGR rat offspring (62). Simultaneously, the expression of cerebral chromatin-affecting enzymes DNA methyltransferase 1 and methyl-CpG binding protein 2 were decreased in neonatal IUGR rats, with the mRNA abundances of 50% and 38% the control values, respectively (62).

DISCUSSION

From the above evidence it is clear that the effects of intrauterine growth restriction may have on the long-term health and well-being of infants are extensive. Diabetes, hypertension, and kidney dysfunction in growth restricted offspring are the most common diseases that were shown to be related to IUGR. Moreover, as the frequency of this pregnancy complication and its associated diseases is high, especially in developing countries, there is a need to determine the mechanisms of how aberrant phenotypes are programmed and transferred to subsequent generations. In comparison to humans, the examination of tissues and organs in animals is more accessible for scientists, ethically. Therefore, the proposed potential mechanisms for the diseases' multigenerational transmission will come from in-depth studies of animal experimental models. Additionally, sex-specific expression of the diseases' phenotypic outcomes

was also observed in animal offspring. Therefore, further assessments are required to determine whether epigenetic mechanisms are responsible for the sex-specific differences of IUGR related diseases. Subsequently, future studies may focus on investigating similar mechanisms and markers in humans, which will help identify people who are at risk and/or identify potential prevention strategies for these diseases.

AUTHOR CONTRIBUTIONS

TD performed the literature search, interpreted the data, and wrote the manuscript. TB-M critically revised the manuscript and supervised the project. LA critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Genome-Wide Placental Gene Methylations in Gestational Diabetes Mellitus, Fetal Growth and Metabolic Health Biomarkers in Cord Blood

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Gestational diabetes mellitus (GDM) "program" an elevated risk of metabolic syndrome in the offspring. Epigenetic alterations are a suspected mechanism. GDM has been associated with placental DNA methylation changes in some epigenome-wide association studies. It remains unclear which genes or pathways are affected, and whether any placental differential gene methylations are correlated to fetal growth or circulating metabolic health biomarkers. In an epigenome-wide association study using the Infinium MethylationEPIC Beadchip, we sought to identify genome-wide placental differentially methylated genes and enriched pathways in GDM, and to assess the correlations with fetal growth and metabolic health biomarkers in cord blood. The study samples were 30 pairs of term placentas in GDM vs. euglycemic pregnancies (controls) matched by infant sex and gestational age at delivery in the Shanghai Birth Cohort. Cord blood metabolic health biomarkers included insulin, C-peptide, proinsulin, IGF-I, IGF-II, leptin and adiponectin. Adjusting for maternal age, pre-pregnancy BMI, parity, mode of delivery and placental cell type heterogeneity, 256 differentially methylated positions (DMPs,130 hypermethylated and 126 hypomethylated) were detected between GDM and control groups accounting for multiple tests with false discovery rate <0.05 and beta-value difference >0.05. WSCD2 was identified as a differentially methylated gene in both site- and region-level analyses. We validated 7 hypermethylated (CYP1A2, GFRA1, HDAC4, LIMS2, NAV3, PAX6, UPK1B) and 10 hypomethylated (DPP10, CPLX1, CSMD2, GPR133, NRXN1, PCSK9, PENK, PRDM16, PTPRN2, TNXB) genes reported

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in previous epigenome-wide association studies. We did not find any enriched pathway accounting for multiple tests. DMPs in 11 genes (CYP2D7P1, PCDHB15, ERG, SIRPB1, DKK2, RAPGEF5, CACNA2D4, PCSK9, TSNARE1, CADM2, KCNAB2) were correlated with birth weight (z score) accounting for multiple tests. There were no significant correlations between placental gene methylations and cord blood biomarkers. In conclusions, GDM was associated with DNA methylation changes in a number of placental genes, but these placental gene methylations were uncorrelated to the observed metabolic health biomarkers (fetal growth factors, leptin and adiponectin) in cord blood. We validated 17 differentially methylated placental genes in GDM, and identified 11 differentially methylated genes relevant to fetal growth.

Keywords: GDM, fetal growth, placental DNA methylations, cord blood biomarkers, birth weight

INTRODUCTION

Gestational diabetes mellitus (GDM) is a common pregnancy complication (1). The offspring of GDM mothers are at an increased risk of metabolic syndrome (2). The mechanisms remain unclear. Epigenetic alterations are a plausible mechanism in such fetal "programming".

Placenta is a fetal organ for nutrient and waste exchanges between the mother and the fetus, and plays a pivotal role in fetal growth and development (3). A number of studies have reported placental epigenetic changes in maternal hyperglycemia *via* candidate gene approach measuring proximal promoter region methylation levels in metabolic health-relevant genes (e.g. leptin, adiponectin, lipoprotein lipase) (4–10). However, later epigenome-wide association studies failed to confirm these findings (11–17). Furthermore, there is a lack of consistent findings in epigenome-wide association studies, probably partly due to relatively small sample sizes and differences in study population, data calibration or statistical analysis method, and definition of GDM (16). Some studies have reported altered methylations in some pathways related to metabolic diseases or fetal development (11, 12, 14, 17), suggesting that placental epigenetic dysregulation may reflect fetal programming effects. We are aware of only one study controlling for placental cell heterogeneity in placental epigenome-wide association analyses in GDM (15). The placental methylome represents a combination of signals from different cell types, and cell type composition/variation (18) would affect gene-level methylation data. Little is known about whether any GDM associated differentially methylated genes are relevant to fetal growth or circulating (cord blood) metabolic health biomarkers.

In the present study, we sought to assess GDM-associated genome-wide DNA methylation changes to identify replicable differentially methylated genes and enriched pathways vs. the literature. We also sought to examine the biological significance of differentially methylated genes through assessing the correlations with fetal growth (birth weight) and metabolic health biomarkers in cord blood.

Abbreviations: GDM, gestational diabetes mellitus; BMI: Body mass index; OGTT, oral glucose tolerance test; SBC, Shanghai Birth Cohort; IADPSG, International Association of Diabetes and Pregnancy Study Groups; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IPA, Ingenuity pathway analysis; CI, confidence interval; SD, standard deviation.

METHODS

Subjects, Data and Specimens

We randomly sampled 30 pairs of GDM and controls in term births with placenta and cord blood specimens available for assays in the Shanghai Birth Cohort (SBC) (19, 20). Briefly, the SBC recruited 4127 women during preconception and early pregnancy care visits during 2013-2016 in six university-affiliated tertiary obstetric care hospitals in Shanghai. Women were followed up at the first, second and third trimesters of pregnancy and delivery, with data and specimens collected at each study visit. The project was approved by the research ethics committees of Xinhua Hospital, Shanghai (the coordination center, ref no. M2013-010) and all participating hospitals.

All participants received a 75-g oral glucose tolerance test (OGTT) at 24-28 weeks of gestation, and GDM was diagnosed according to the International Association of Diabetes and Pregnancy Study Group (IADPSG) criteria (blood glucose concentration >=anyone of the following thresholds: fasting 5.1 mmol/L, 1-hour 10.0 mmol/L and 2-hour 8.5 mmol/L) (21). The present study included 30 GDM (5 with insulin treatment, 25 with dietary/lifestyle interventions) and 30 euglycemic controls matched by fetal sex and gestational age at delivery (within 1 week). All cases and controls were term births with normal Apgar score (>7), and all mothers were free of severe chronic diseases before pregnancy (e.g., essential hypertension, chronic diabetes) or severe pregnancy complications (e.g. preeclampsia).

Birth weight was standardized to z score according to the 2015 Chinese sex- and gestational age-specific birth weight standards (22). Maternal pre-pregnancy body mass index (BMI) (kg/m^2) was categorized by Chinese standards (<18.5 underweight; 18.5-23.9 normal weight; 24.0+ overweight/obesity) (23). Other maternal and infant characteristics included maternal ethnicity (all Han Chinese), age (years), education (university: yes/no), parity (primiparity: yes/no), family history of diabetes in first-degree relatives (yes/no),

mode of delivery (cesarean section/vaginal), infant sex and gestational age (week) at birth.

Cord blood and placenta specimens were collected immediately after delivery. Cord blood samples were centrifuged within 2 hours of specimen collection at 4°C, 4000 r/min for 10 min. Serum (without any anticoagulant) and EDTA plasma aliquots were stored at -80°C until assays. Each of the four placenta quadrants was sampled approximately 1.5 cm away from the umbilical cord insertion from the fetal side of the placenta. Fetal membrane and visible large vessels were removed, and phosphate-buffered saline (PBS) was used to wash placenta samples before separating into maternal- and fetal-side samples. Samples were stored at -80°C until DNA extraction. DNA was extracted from fetal-side placental samples using DNeasy Blood & Tissue Kit (Qiagen, Cat No. 69504). Purity was examined by A_{260}:A_{280} ratio which ranged between 1.87 and 1.90.

Epigenome-Wide DNA Methylation Measurements

Placental DNA methylations at more than 850,000 CpG sites across the genome were measured by Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA). Samples were randomly placed in different slides (total 12) in the assays. Package "minfi" was used to import and preprocess raw methylation data in R. For all the samples, the proportion of CpG sites with detection P value >0.01 was less than 5%. Thus, all samples were included in subsequent data analyses. We excluded 2677 CpG sites with detection P value >0.01 in more than 5% of all the samples. Functional normalization was applied to remove between-array (unwanted) variations using control probes (24). We excluded 41710 CpG sites with bead count less than 3 in more than 5% of all samples, 18487 annotated to sex chromosomes, 83460 SNPs inside the probe body, in the CpG interrogation, or at the single nucleotide extension with a minor allele frequency of ≥0.05, 33202 suspected cross-reactive sites (25), and 2301 non-CpG sites, leaving 684022 CpG sites in further data analysis. Beta-mixture quantile dilation (BMIQ) was then applied to adjust for type-2 probe bias (26). We excluded sites with average methylation levels <5% or >95% (n=183267), as extreme β values tended to have low reproducibility (27), and small to moderate changes in methylation levels may not have any significant biological implication at both extremes. A total of 500755 CpG sites were retained in the final epigenome-wide association analysis. Potential bias due to different slides was adjusted for by the ComBat function in sva package (28). The density plot did not reveal significant differences in beta value distributions between GDM and control groups (**Figure S1**). Beta values were transformed to M values using lumi package for differential methylation analysis since M values had better statistical performance (29). Beta values were presented for quantifying the differences in methylation levels between groups for ease of data interpretation.

Bisulfite Sequencing Validation Study

We sought to validate a few selected DMPs with relatively larger methylation differences between GDM and control groups in the epigenome wide association analysis. The study subjects were an independent random sample of 47 pairs of GDM and controls from the Shanghai Birth Cohort. 4 DMPs (CpG sites) were chosen as the corresponding delta betas were in the top 30 DMPs and the corresponding genes have been related to glucose homeostasis (30, 31). These CpG sites were annotated to WSC Domain Containing 2 (WSCD2), phosphodiesterase 1C (PDE1C), and protocadherin Beta 15 (PCDHB15). DNA was sodium-bisulfite treated (EZ DNA Methylation-Lighting) and PCR-amplified with primers designed by PyroMark Assay Design software (version 2.0; Qiagen). Pyrosequencing was performed using PyroMark Q48 (Qiagen, appendix – Methodology in the pyrosequencing study).

Biochemical Assays

Cord serum insulin and insulin-like growth factor 1 (IGF-I) concentrations were measured by chemiluminescent assays (ADVIA Centaur and Immulite2000, SIEMENS, Germany). Cord plasma C-peptide and proinsulin were measured by enzyme-linked immunosorbent assay (ELISA) kits from Mercodia (Uppsala, SE). IGF-II was measured by an ELISA kit from R&D system (Minnesota, USA). Plasma high-molecular-weight (HMW) and total adiponectin concentrations were measured by an ELISA kit from ALPCO (Salem, NH, USA), and plasma leptin by an ELISA kit from Invitrogen (Carlsbad, CA, USA), respectively. The detection limits were 3.5 pmol/l for insulin, 25 pmol/L for C-peptide, 1.7 pmol/l for proinsulin, 25 ng/ml for IGF-I, 1.88 pg/ml for IGF-II, 0.034 ng/ml for HMW and total adiponectin, and 3.5 pg/ml for leptin, respectively. Intra-assay and inter-assay coefficients of variation were in the ranges 0.4-13.5% (32, 33). The laboratory technicians were blinded to the clinical status of study participants.

Statistical Analysis

All analyses were conducted in R Studio (version: 2021.09.1). The associations between GDM status and gene DNA methylation levels (M-values) were assessed by lmFit function in R limma package. For placental cell types, we used ReFactor to select the number of principal components accounting for a substantial proportion of the variance in the data, and then compute the selected 4 components in adjusting for cell-type heterogeneity in differential methylation position (DMP) analyses (34). The comparisons were adjusted for important covariates including maternal age, pre-pregnancy body mass index, primiparity (yes/no), infant sex, gestational age at birth and mode of delivery (vaginal/caesarean section). To minimize false discovery findings, p values were adjusted according to Benjamini and Hochberg's method accounting for multiple tests. DMPs were selected at false discovery rate (FDR) threshold of 0.05 and absolute methylation difference (delta beta) >0.05 (to identify "true" DMPs with differences that are unlikely be measurement errors) (16). To explore the functional roles of the DMPs, we performed Ingenuity Pathway Analysis to annotate the most significant canonical pathways.

Differentially methylated regions (DMRs) were identified using comb-p (35) and DMRcate package (36). Comb-p deals with autocorrelations in neighboring P values and reports

region-based P values with Sidak correction for multiple tests. P value $<10^{-3}$ was set to start a region and a distance of 200 bp was selected to extend the region in the presence of another P value $<10^{-3}$. DMRcate package identified the most differentially methylated regions based on tunable kernel smoothing of the signal of methylation changes (36). As recommended by the authors, a bandwidth of 1000 nucleotides and a scaling factor of 2 were used. Significant DMRs were selected at FDR <0.05.

Partial correlation analyses were conducted to examine the associations with cord blood metabolic health biomarkers (insulin, proinsulin, C-peptide, IGF-I, IGF-II, leptin, adiponectin) adjusting for gestational age at birth. First, we assessed the correlations with DNA methylation levels in the respective specific genes (LEP, ADIPOQ, IGF1, INS-IGF2). Of the 500755 CpG sites, 21 sites were annotated to LEP (chr7: 127876829-127894849), 12 sites to ADIPOQ (chr3: 186559147-186575325), 12 sites to IGF1 (chr12: 102795843-102875301) and 119 sites to INS/INS-IGF2/IGF2 (chr11: 2150687- 2183864). For each gene, mean methylation levels were computed for moderately/highly correlated sites (r>0.5). Benjamini-Hochberg method was used in calculating the adjusted P values accounting for multiple tests. Second, we assessed the correlations of GDM-associated DMPs with birth weight (z score) and cord blood metabolic health biomarkers (in log-transformed data). CpG sites with crude P values <0.05 were presented along with the Benjamini-Hochberg adjusted P values accounting for multiple tests.

RESULTS

Characteristics of Study Subjects

Comparing GDM vs. controls (n=30 pairs), women with GDM had higher pre-pregnancy BMI (24.4 ± 3.5 vs. 21.2 ± 3.8 kg/m^2), and were more likely to be overweight or obese (57.7% vs 18.5%), or to have a caesarean section delivery (73.3% vs. 26.7%) (**Table 1**). As expected, women with GDM had higher fasting, 1h and 2h blood glucose levels in the 75-OGTT tests. All women were non-smokers. There were no significant differences in maternal age, education, parity, family history of diabetes, alcohol drinking during pregnancy and gestational weight gain. Comparing the infants of women with GDM vs. euglycemic pregnancies, birth weight z scores were substantially higher (1.23 ± 1.39 vs. 0.13 ± 0.66).

Cord plasma high molecular weight adiponectin concentrations were lower in GDM vs. controls (14.9 ± 8.9 vs. 20.5 ± 10.4 μg/ml, P=0.015), while the differences in cord blood insulin, C-peptide, proinsulin, IGF-I, IGF-II and leptin concentrations were not statistically significant.

Differentially Methylated Positions

Adjusting for maternal age, pre-pregnancy BMI, parity, infant sex, gestational age, mode of delivery and the four principal components representing placental cell type proportions, at FDR <0.05 and delta beta >0.05, we identified 256 DMPs (130 hypermethylated and 126 hypomethylated) between GDM and control groups. These loci were distributed over 133 genes (71 genes in hypermethylated loci, 64 genes in hypomethylated loci; 3 genes were in both) (**Table S3**). Epigenome-wide association results were shown in **Figure S2**. The mean ± SD of the methylation differences between GDM and control groups were 7.5 ± 2.7% and 7.2 ± 2.5% for hypermethylated and hypomethylated loci, respectively.

The top 30 DMPs (15 hypermethylated, 15 hypomethylated) are presented in **Table 2**. The β value differences (Δβ) in the 15 hypermethylated sites range from 0.11 to 0.18. These loci were annotated to 12 genes (PCDHB15, DKK2, FAM65B, CYP26C1, MIATNB, ERG, CADM2, GFRA1, DPYD, HCN1, C14orf39, and SLC9A9). The Δβ of the top 15 hypomethylated sites range from -0.18 to -0.10, and these sites were annotated to 8 genes (DPP10, PENK, TSNARE1, WSCD2, LOC102723828, NRXN1, PDE1C and MIR124-2).

Pyrosequencing Validation Study in an Independent Sample

The differences in methylation levels between GDM and control groups in the pyrosequencing validation study (n=47 GDM-control pairs) and Infinium MethylationEPIC BeadChip (850K) study are in the same direction for all the four DMPs in the three genes (WSCD2, PDE1C, and PCDHB15) (**Table S1**), although the methylation differences between the two groups did not reach statistical significance in the pyrosequencing study.

Validated Differentially Methylated Genes vs. the Literature

The validated placental differentially methylated genes vs. the literature are listed in **Table S2**. Our MethylationEPIC BeadChip data validated 7 hypermethylated genes (CYP2D7P1, GFRA1, HDAC4, LIMS2, NAV3, PAX6 and UPK1B), and 10 hypomethylated genes (DPP10, CPLX1, CSMD2, GPR133, NRXN1, PCSK9, PENK, PRDM16, PTPRN2 and TNXB) reported in previous placenta epigenome-wide association studies in GDM (11, 13, 16).

Ingenuity Pathway Analysis

IPA analysis of DMPs identified 10 canonical pathways with Fisher's exact test crude P<0.05 (**Table 3**). However, none of the pathways achieved statistical significance after correction for multiple tests. Differentially methylated genes were most enriched in bladder cancer signalling (4 genes, crude P=0.005), followed by GABA receptor signalling (4 genes, crude P=0.008) and uracil degradation II (reductive) (1 gene, crude P=0.023).

Differentially Methylated Regions

In search of genomic regions where methylation levels differ between GDM and control groups, comb-p identified 4 DMRs (**Table S4**) with Sidak corrected P<0.05. Two DMRs were annotated to PCSK9 and WSCD2D, while the other two DMRs were located in the OpenSea area. DMRcate identified 42 DMRs at FDR <0.05 that were annotated to 29 genes spanning 2 to 13 CpG sites (**Table S5**). The most significant region was located in the promoter and the first exon of WSCD2 in chromosome 12, containing 13 CpG sites. Two overlapped DMRs were identified by both DMRcate and comb-p. One DMR (Chr12:108522704-108524345) was located in the WSCD2 promoter and the other

TABLE 1 | Maternal and neonatal characteristics in a matched study of 30 pairs of term placentas in gestational diabetes mellitus (GDM) vs. euglycemic (control) pregnancies in Shanghai birth cohort.

	GDM (n=30)	Control (n=30)	P*
Mothers			
Age	29.8 ± 4.1	29.8 ± 3.2	0.999
>35 years	4 (13.3)	2 (6.7)	0.335
Alcohol drinking in pregnancy	2 (7.7)	7 (25.9)	0.079
Family history of diabetes	6 (20.0)	2 (6.7)	0.127
Education, university	18 (60.0)	20 (66.7)	0.592
Primiparity	26 (86.7)	24 (80.0)	0.757
Prepregnancy BMI (kg/m^2)	24.4 ± 3.48	21.2 ± 3.78	**0.002**
BMI group			**0.023**
<18.5	1 (3.8)	5 (18.5)	
18.5-24.0	10 (38.5)	17 (63.0)	
24.0+	15 (57.7)	5 (18.5)	
Gestational weight gain (kg)	14.9 ± 4.8	15.9 ± 4.9	0.475
Z score	0.17 ± 0.81	0.19 ± 0.99	0.920
Caesarean section	22 (73.3)	8 (26.7)	**<0.001**
75 g OGTT glucose (mmol/L)			
Fasting	4.9 ± 0.6	4.4 ± 0.4	**<0.001**
1-hour	9.8 ± 1.7	7.0 ± 1.5	**<0.001**
2-hour	7.9 ± 2.0	6.0 ± 1.2	**<0.001**
HbA1c (%) at 32-35 weeks	5.3 ± 0.4	5.1 ± 0.3	0.061
Newborns			
Birth weight (g)	3814 ± 524	3390 ± 264	**<0.001**
Z score	1.23 ± 1.39	0.13 ± 0.66	**<0.001**
Birth length (cm)	50.5 ± 1.14	50.05 ± 0.68	0.076
Z score	0.51 ± 1.13	0.03 ± 0.71	0.060
Sex, boy	17 (56.7)	17 (56.7)	1.000
Gestational age at delivery	39.4 ± 0.68	39.6 ± 0.80	0.185
Cord blood biomarkers			
Insulin (pmol/L)	32.7 ± 28.9	40.1 ± 43.4	0.716
C-peptide (pmol/L)	284.5 ± 170.6	286.8 ± 161.1	0.994
Proinsulin (pmol/L)	26.9 ± 22.4	24.4 ± 22.1	0.920
Leptin (ng/ml)	9.3 ± 7.7	9.4 ± 6.7	0.407
Adiponectin-HMW (μg/ml)	14.9 ± 8.9	20.5 ± 10.4	**0.018**
Adiponectin-Total (μg/ml)	32.8 ± 14.9	40.6 ± 18.4	0.073
IGF-I (ng/ml)	81.6 ± 27.9	68.7 ± 22.9	0.083
IGF-II (ng/ml)	198.5 ± 28.3	191.4 ± 32.8	0.335

Data presented are mean ± SD or n (%); HMW, high-molecular-weight.
**P values for differences between the two groups; P values in bold, P<0.05.*

(Chr5: 92275312-92275387) in the OpenSea area (**Table 4**). The methylation levels in the GDM group were lower in the WSCD2 promoter region.

Gene-Specific Correlations Between Placental DNA Methylations and Cord Blood Biomarkers

Table S6 presents gene-specific correlations of placental gene DNA methylations with cord blood biomarkers. The total numbers of sites in the Pearson partial correlation analyses were 17 for LEP, 12 for ADIPOQ, 7 for IGF1 and 71 for INS/INS-IGF2/IGF2 genes, respectively. For LEP, methylation at cg05136031 was positively correlated with cord blood leptin concentration (r=0.28, crude P=0.033). For INS-IGF2/IGF2/INS, methylation levels in two CpG sites (cg17434309, cg10650127) were positively correlated with cord blood insulin (r=0.32 and P=0.009, r=0.29 and P=0.028, respectively). Methylation at cg17434309 was correlated with C-peptide. Methylation levels in one site and one region (cg13670288, mean of cg27331871 and cg25742037) were negatively correlated with cord blood proinsulin. Methylation levels in three CpG sites and one region (cg23889607, cg24366657, cg25163476, mean of cg02749887 and cg21574853) were positively correlated with IGF2. However, all these correlations did not reach statistical significance after correction for multiple comparisons.

Correlations of Placental DMPs With Birth Weight and Cord Blood Biomarkers

Among the 256 DMPs, 38 DMPs (34 genes) were correlated with birth weight (z score) at crude P<0.05, and the correlations remained statistically significant for 12 DMPs (11 genes) after BH correction for multiple tests (**Table 5**). Methylations in seven genes (PCDHB15, DKK2, ERG, CADM2, CYP2D7P1, SIRPB1, KCNAB2) were positively correlated with birth weight with BH adjusted P <0.05. Methylations in four genes (RAPGEF5, CACNA2D4, PCSK9, TSNARE1) were negatively correlated with birth weight with BH adjusted P<0.05.

In regression analyses, the positive association between GDM and birth weight (β=1.10, P<0.01) became non-significant after

TABLE 2 | Top 30 differentially methylated positions (15 hypermethylated and 15 hypomethylated DMPs) in placental DNAs in GDM vs. euglycemic (control) groups.

CpG site	Chr	Gene	Gene group	Beta (GDM)	Beta (Control)	Delta beta	FDR
Hypermethylated							
cg06295987	chr3			0.323	0.14	0.183	0.007
cg26380443	chr5	PCDHB15	1stExon	0.388	0.212	0.176	0.046
cg17010273	chr4	DKK2	TSS1500	0.373	0.205	0.169	0.019
cg03975200	chr6	FAM65B	TSS1500	0.711	0.546	0.165	0.047
cg07204602	chr10	CYP26C1	TSS1500	0.752	0.601	0.152	0.048
cg11423008	chr22	MIATNB	Body	0.775	0.627	0.149	0.024
cg15480311	chr21	ERG	Body; 5'UTR	0.519	0.391	0.128	0.039
cg25429559	chr3	CADM2	5'UTR;Body	0.858	0.732	0.126	0.041
cg06039355	chr10	GFRA1	5'UTR;TSS1500	0.441	0.321	0.12	0.007
cg26836456	chr1	DPYD	TSS1500	0.507	0.388	0.119	0.049
cg18414238	chr5	HCN1	Body	0.681	0.569	0.112	0.048
cg12189551	chr14	C14orf39	TSS200	0.261	0.15	0.112	0.042
cg16079012	chr3	SLC9A9	Body	0.744	0.635	0.109	0.049
cg17943876	chr5			0.552	0.445	0.107	0.007
cg05684050	chr5			0.775	0.67	0.106	0.04
Hypomethylated							
cg16863522	chr2	DPP10	3'UTR	0.274	0.454	-0.18	0.017
cg27531336	chr8	PENK	TSS1500	0.276	0.433	-0.157	0.037
cg13112267	chr8	TSNARE1	Body	0.642	0.799	-0.157	0.046
cg13713677	chr12	WSCD2	TSS200	0.182	0.334	-0.152	0.028
cg05041928	chr4	LOC102723828	Body	0.561	0.697	-0.136	0.036
cg08664343	chr21			0.563	0.693	-0.13	0.028
cg26852437	chr11			0.561	0.69	-0.128	0.012
cg05581275	chr2	DPP10	Body	0.291	0.406	-0.115	0.012
cg13594075	chr2	NRXN	TSS200	0.411	0.526	-0.114	0.026
cg01097881	chr12	WSCD2	TSS200	0.25	0.362	-0.112	0.012
cg19502018	chr7	PDE1C	Body	0.583	0.695	-0.111	0.011
cg04831599	chr8			0.377	0.486	-0.109	0.03
cg24118713	chr12			0.634	0.741	-0.106	0.01
cg05474726	chr8	MIR124-2	TSS200	0.269	0.375	-0.106	0.04
cg10104921	chr4			0.491	0.596	-0.104	0.028

FDR, false discovery rate accounting for multiple tests.

adjusting for the methylation levels in these 38 DMPs (β=-0.76, P=0.42). These 11 genes were enriched in nNOS signaling in skeletal muscle cells (Fisher's exact crude P=0.024), netrin signaling (P=0.037), maturity onset diabetes of young signaling (P=0.039), and FcγRIIB signaling in B lymphocytes (P=0.042).

Placental gene DMPs that were significantly correlated with one or more cord blood biomarkers (leptin, total and HMW-adiponectin, insulin, proinsulin, C-peptide, IGF1 and IGF2) at crude P <0.05 are presented in **Table S7**. Notably, PAX6 (cg09382096) methylation was negatively correlated with cord blood leptin (r=-0.34, P=0.01), total adiponectin (r=-0.30, P=0.02) and HMW-adiponectin (r=-0.39, P=0.003). SLC9A3 (cg02689506) methylation was negatively correlated with cord blood leptin (r=-0.31, P=0.02), total adiponectin (r=-0.32, P=0.014) and HMW-adiponectin (r=-0.44, P=0.001). WSCD2 (cg15873673) methylation was positively correlated with cord blood total adiponectin (r=0.30, P=0.02), HMW-adiponectin (r=0.28, P=0.036), but negatively correlated with insulin (r: -0.29 to -0.32, P<0.05) and C-peptide (r: -0.29 to -0.31, P<0.05). FGF19 (cg18210732) methylation was negatively correlated with cord blood leptin (r=-0.30, P=0.02) and HMW-adiponectin (r=-0.28, P=0.034). However, none of these correlations reached statistical significance after correction for multiple tests.

TABLE 3 | Canonical pathways of differentially methylated positions (DMPs) in placental genes in GDM vs. control groups.

Pathway	P*	Overlapped genes (ratio)
Bladder Cancer Signaling	0.005	4/114 (0.035)
GABA Receptor Signaling	0.008	4/130 (0.0308)
Uracil Degradation II (Reductive)	0.023	1/4 (0.25)
Thymine Degradation	0.023	1/4 (0.25)
CREB Signaling in Neurons	0.025	8/591 (0.0135)
tRNA Splicing	0.028	2/44 (0.0455)
Gustation Pathway	0.030	4/197 (0.0203)
Amyotrophic Lateral Sclerosis Signaling	0.031	3/114 (0.0263)
Cardiac Hypertrophy Signaling (Enhanced)	0.039	7/527 (0.0133)
cAMP-mediated signaling	0.047	4/226 (0.0177)

**Crude p value from Fisher's exact test.*

TABLE 4 | Differentially methylated regions (DMRs, GDM vs. control groups) identified by both comb-p and DMRcate package in region-level data analyses in R.

DMR	CpGs comprising the DMR	Direction of association	Gene	Gene group/Relation to island
comb-p				
Chr 5:92275335-92275388	cg21937916, cg19564029	+		OpenSea
Chr 12:108523395-108523473	cg01097881, cg13236378, cg05062612, cg26373942, cg06326926	–	WSCD2	TSS200/Island
DMRcate				
Chr 5: 92275312-92275387	cg14411504, cg19564029, cg21937916	+		OpenSea
chr12:108522704-108524345	cg12728312, cg22330512, cg13713677, cg08047802, cg03111482, cg01097881, cg13236378, cg05062612, cg26373942, cg06326926, cg15873673, cg10419678 cg03626024	–	WSCD2	TSS1500, TSS200, 5'UTR,1stExon/N_Shore, Island, S_Shore

GDM, gestational diabetes mellitus.

DISCUSSION

Main Findings

This study identified 256 GDM-associated placental DNA DMPs, and validated 17 differentially methylated genes reported in previous epigenome-wide association studies. The WSCD2 gene was identified as differentially methylated in both site- and region-level analyses. We discovered methylations in 11 placental genes relevant to fetal growth, but none placental gene methylations were correlated to the observed metabolic health biomarkers (fetal growth factors, leptin and adiponectin) in cord blood accounting for multiple tests.

Differentially Methylated Positions

We detected 256 DMPs localized on 133 genes, and validated 17 differentially methylated genes (CYP1A2, GFRA1, HDAC4, LIMS2, NAV3, PAX6, UPK1B, DPP10, CPLX1, CSMD2, GPR133, NRXN1, PCSK9, PENK, PRDM16, PTPRN2, TNXB) reported in the literature (11, 13, 16). Several genes have been implicated in glucose metabolism and merit comments. GFRA1 has been related to β-cell proliferation and islet repairing (37). GFRA1 was hypermethylated in GDM placentas in a previous study (13). We observed that a locus of PAX6 was hypermethylated in GDM placentas, consistent with a previous study (16). PAX6 may be involved in the regulation of glucose-insulin homeostasis (38, 39). PCSK9 and PTPRN2 have been associated with lipoprotein metabolism and insulin release, respectively (40–42), and were hypomethylated in our and previous epigenome-wide studies (13, 16).

Our and other studies demonstrated substantial placental epigenetic changes in GDM, but only 17 replicable differentially methylated genes vs. the literature. This may be partly attributable to differences in sample size, GDM definition, and cell heterogeneity in placental sample tissues. DNA methylation changes by high-throughput methods in bulk placental tissues should be interpreted with caution. Validation data from different study cohorts may provide important clues on the true altered gene methylations. Ethnicity may be a factor influencing the reproducibility of epigenetic data. All our study subjects were Chinese. It is noteworthy that our data have validated 17 differentially methylated genes in previous reports in Caucasians (11, 13, 16).

The placenta secretes numerous hormones and factors into maternal circulation affecting maternal metabolic health. It is therefore possible that these GDM associated placental differentially methylated genes may be involved in the development of GDM and related complications. Further studies are warranted to understand the potential roles of these genes in the pathophysiology of GDM, and whether the relevant proteins could be promising molecular targets in the prevention of GDM and related complications.

Bisulfite-Pyrosequencing Validation Study

Bisulfite-pyrosequencing method is the golden standard to measure DNA methylation. We used an independent sample (n=47 GDM/control pairs) in the pyrosequencing validation study of four DMPs annotated to three genes (WSCD2, PCDHB15, and PDE1C). Although the methylation level differences between GDM and controls in the pyrosequencing validation study were all in the same pattern as the 850K BeadChip discovery study, all the differences were not statistically significant in the pyrosequencing validation study. Possible reasons may be partly related to the complex nature of placental tissues; methylation data were likely influenced by cell type composition which was difficult to adequately account for.

Differentially Methylated Regions

Two DMRs were identified in both comb-p (35) and DMRcate (36) analyses in our study. One DMR was located in the promoter of WSC Domain Containing 2 (WSCD2). Placenta differentially methylated DNA regions in GDM were not assessed in previous studies. A total of 8 CpGs in the WSCD2 gene were identified as hypomethylated DMPs – a consistent finding between region- and site-based data analysesWSCD2 functions as an integral component of the membrane. A single nucleotide polymorphism (rs59849892) in the intronic region of WSCD2 has been linked to insulin sensitivity (43). A recent study reported that WSCD2 expression in human islet was positively correlated with insulin secretion and negatively correlated with HbA1c, suggesting possible

TABLE 5 | Correlations of birth weight (z score) with differentially methylated genes.

Gene	probeID	r	P	BH corrected P*
CYP2D7P1	cg26169700	0.432	0.00056	0.04096
PCDHB15	cg26380443	0.420	0.00084	0.04096
ERG	cg15480311	0.416	0.00096	0.04096
SIRPB1	cg11330406	0.414	0.001	0.04096
DKK2	cg17010273	0.401	0.00152	0.04096
RAPGEF5	cg24092655	-0.399	0.00157	0.04096
RAPGEF5	cg20583679	-0.389	0.00213	0.04651
CACNA2D4	cg10719390	-0.388	0.00218	0.04651
PCSK9	cg17167852	-0.382	0.00258	0.04722
TSNARE1	cg13112267	-0.378	0.00289	0.04722
CADM2	cg25429559	0.375	0.00317	0.04722
KCNAB2	cg00813560	0.371	0.00352	0.04743
DPYSL2	cg06601131	0.362	0.00449	0.05225
NME7	cg16468828	0.348	0.00647	0.06370
LOC101927292	cg05401617	-0.341	0.00759	0.06939
LAIR2	cg20313638	0.34	0.00795	0.07018
PDE1C	cg19502018	-0.334	0.00919	0.07842
CCT4	cg12168100	0.331	0.0098	0.08093
CD200R1L	cg24025902	-0.328	0.01046	0.08114
UPK1B	cg26433505	0.317	0.01364	0.09563
KIAA1429	cg17966245	0.315	0.01419	0.09563
MIATNB	cg11423008	0.312	0.01512	0.09563
C3P1	cg12272488	0.312	0.01537	0.09563
MBP	cg12979350	-0.311	0.01569	0.09563
NAV3	cg20297402	0.307	0.01708	0.10169
PLIN5	cg02593507	-0.299	0.02038	0.11253
PCSK9	cg09072162	-0.294	0.02244	0.11489
MIATNB	cg07281538	0.289	0.02504	0.12569
C4orf37	cg08746785	0.285	0.02716	0.13119
OBSCN	cg02281446	-0.280	0.03016	0.13811
C1orf114	cg06783102	-0.279	0.03071	0.13811
NACC2	cg24597547	-0.277	0.03197	0.14111
PHACTR3	cg08481633	0.269	0.03744	0.15819
GABRA5	cg00796569	0.264	0.04151	0.16604
CFAP70	cg24284700	0.261	0.04364	0.17187
PTPRN2	cg24764310	-0.260	0.04461	0.17303
NACC2	cg15344596	-0.259	0.04533	0.17320
FRMD6	cg09376996	0.256	0.04875	0.18353

**P value with Benjamini-Hochberg correction for multiple tests.*

hypermethylation in the WSCD2 gene in islet cells in diabetes status and a role of WSCD2 in glucose metabolism (30). However, the placental WSCD2 gene was observed to be hypomethylated in GDM, underscoring the importance of tissue specificity and heterogeneity considerations in methylation data interpretation. The other DMR was not annotated to any gene, and its implications remain to be understood. In contrast, Howe et al. identified two different DMRs in cord blood DNA methylations in GDM using both comb-p and DMRcate in a pooled data analysis of 3677 mother-infant pairs from 7 pregnancy cohorts (44). One DMR was located in the promoter of OR2L13 which has been associated with autism spectrum disorders, and the other was located in the gene body of CYP2E1 which has been implicated in type 1 and type 2 diabetes (44). The discrepant findings could be due to the different tissues in measuring DNA methylations (cord blood vs placenta in our study).

Canonical Pathways

Our study identified 9 canonical pathways in the IPA analysis with crude P<0.05 in the Fisher's exact test. These pathways are not overlapping with those reported pathways in previous studies, such as the Wnt signaling (12, 17), MAPK signaling (12, 14, 17), or insulin signaling pathway (14). However, we did not find all statistically significant pathway after correction for multiple tests. The lack of significance could result from a relatively small number of differentially methylated genes (n=133) for identifying the enriched pathways. It should be noted that previous studies used Fisher's exact test only in the IPA analysis, without correction for multiple tests. The lack of replicable findings concerning the pathways in our study vs. the literature underscores the need for more and larger studies, and the need for standardized analysis approaches.

Correlations Between DMPs and Birth Weight or Cord Blood Biomarkers

We observed that methylation levels in 11 placental genes were significantly correlated to birth weight (z score) after correction for multiple tests. Birth weight may partly reflect the impact of GDM on the fetus (45). Our results suggest placental epigenetic alterations may play a mediating role in the impact of GDM on

fetal growth. We are aware of only one study on placental DMPs in GDM and birth weight, and the study reported 326 GDM associated genes (11). TSNARE1 was the only common gene to these identified in our study. Surprisingly, DNA methylation levels in the TSNARE1 were positively correlated with birth weight in their study (r=0.47, crude P=0.007) (11), but negatively correlated with birth weight in our study. The inconsistent findings suggest the need for more validation studies. We noted that the previous study did not account for multiple tests in examining these correlations (11).

Of the 11 genes with methylations correlated with fetal growth in our study, methylation levels at the PCSK9 promoter were inversely correlated with birth weight (z score). Consistent with our data, a recent study reported lower cord blood PCSK9 concentrations (could be due to higher methylation levels) in intrauterine growth restricted vs. control newborns (46). PCSK9 may play a role in lipoprotein metabolism (40). Circulating PCSK9 concentrations have been positively correlated with total cholesterol, low-density lipoprotein (LDL) cholesterol, and body mass index (47, 48). DNA methylation levels at cell adhesion molecule 2 (CADM2) were positively correlated with birth weight. CADM2 is predominantly expressed in brain, and regulates energy homeostasis *via* central nervous system (49). CADM2 depletion has been associated with reduced adiposity and improved insulin sensitivity in genetically obese mice models (49). Human and animal studies suggest that CADM2 variations regulate body weight (50, 51). We are unaware of any reports on the other 9 genes associated with fetal growth in our data.

We observed no significant associations between GDM-associated DMPs and cord blood biomarkers after correction for multiple tests. We are unaware of any studies on these correlations. Crude correlation coefficients showed that DNA methylation levels at some loci of WSCD2 were correlated with insulin, C-peptide and HMW-adiponectin concentrations. WSCD2 has been implicated in insulin secretion and insulin sensitivity (30, 43). The crude correlation coefficients showed that DNA methylation levels of PAX6 were correlated with leptin and adiponectin concentrations. PAX6 may regulate insulin transcription (52). However, we did not find any correlation between placental PAX6 and cord blood insulin, C-peptide or proinsulin. FGF19 has been implicated in lipid and glucose metabolism (53). Placental DNA methylation levels in FGF19 were correlated with cord blood leptin and HMW-adiponectin. However, none of these correlations were significant after correction for multiple tests, suggesting the need for more validation studies.

Correlations Between Gene-Specific Placental DNA Methylation and Cord Blood Biomarkers

Adjusting for multiple tests, we did not find any significant correlation between gene-specific placental DNA methylations and the respective cord blood metabolic health biomarkers including insulin, IGF-1, IGF-2, leptin and adiponectin. We observed a trend towards a positive correlation between methylation in a CpG site in the LEP promoter (cg05136031) and cord blood leptin, consistent with a recent study report (54). The cg05136031 methylation has been negatively correlated with BMI (z score) at age 3 years (54). We did not find any correlation between cg15758240 and cord blood leptin, while the previous study reported a negative correlation that might mediate the association between maternal hyperglycemia and cord blood leptinemia (n=262) (54). The non-significant association in our study may be partly attributable to the relatively small sample size (n=60).

Strengths and Limitations

The major strengths include the use of the most recent DNA methylation microarray covering 850K+ CpG sites, and the more robust statistical analysis approach adjusting for placental cell heterogeneity. All study subjects were Chinese, minimizing the potential confounding effects due to ethnic variations in DMPs. The study has limitations. First, the study had a moderate sample size, and we could not rule out the possibility of false positive findings. Large studies are warranted to validate the findings. Sub-group analyses by GDM treatment (insulin/diet) were not possible due to small sample size (only 5 GDM patients with insulin treatment). Second, DNA methylation levels from pyrosequencing validation study did not achieve statistically significant differences between GDM and control groups, underscoring the uncertain nature of discoveries from genome-wide association analyses. Third, maternal lifestyle factors including smoking, physical activity and dietary factors may affect placental gene methylations. In our study, all pregnant women were non-smokers. We did not have data on maternal physical activity and dietary factors. Further studies are warranted to understand whether these factors may confound the associations between GDM and placental gene methylations.

In summary, GDM was associated with altered DNA methylations in a number of placental genes, but these placental gene methylations were uncorrelated to the observed metabolic health biomarkers in cord blood (fetal growth factors, leptin and adiponectin). We validated 17 GDM associated differentially methylated placental genes reported in the literature, and discovered 11 differentially methylated genes relevant to fetal growth.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because access to the deidentified participant research data must be approved by the research ethics board on a case-by-case basis. The EWAS data on placental gene methylations are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE200659. Requests to access the datasets should be directed to Zhong-Cheng Luo (zcluo@lunenfeld.ca) or Fangxiu Ouyang (ouyangfengxiu@xinhuamed.com.cn) for assistance in data access request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the research ethics committees of Xinhua Hospital and all participating hospitals. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Z-CL, J-GF, FL, LB, JZ and FO conceived the study. W-JW, M-NY, RH, TZ, QD, Y-JX, XL, M-YT, HH, FF, J-GF, FL, JZ, FO and Z-CL contributed to the acquisition of research data. WJW and RH conducted the literature review, data analysis and drafted the manuscript. All authors contributed in revising the article critically for important intellectual content, and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.875180/full#supplementary-material

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CASE REPORT
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Case Report: A Novel Truncating Variant of *NR0B1* Presented With X-Linked Late-Onset Adrenal Hypoplasia Congenita With Hypogonadotropic Hypogonadism

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Nuclear receptor subfamily 0 group B member 1 gene (*NR0B1*) encodes an orphan nuclear receptor that plays a critical role in the development and regulation of the adrenal gland and hypothalamic–pituitary–gonadal axis. In this study, we report a novel mutation in *NR0B1* that led to adult-onset adrenal hypoplasia congenita (AHC) and pubertal development failure in a male adult. Clinical examinations revealed hyponatremia, elevated adrenocorticotropic hormone levels, reduced testosterone and gonadotropin levels, and hyper-responses to gonadotropin-releasing hormone and human chorionic gonadotropin stimulation tests. Whole-exome sequencing and Sanger sequencing were performed to identify the potential causes of AHC. Candidate variants were shortlisted based on the X-linked recessive models. Sequence analyses identified a novel hemizygous variant of c.1034delC in exon 1 of *NR0B1* at Xp21.2, resulting in a frameshift mutation and premature stop codon formation. The c.1034delC/p.Pro345Argfs*27 in the *NR0B1* gene was detected in the hemizygous state in affected males and in the heterozygous state in healthy female family carriers. These results expand the clinical features of AHC as well as the mutation profile of the causative gene *NR0B1*. Further studies are needed to elucidate the biological effects of the mutation on the development and function of the adrenal gland and the hypothalamic–pituitary–gonadal axis.

Keywords: adrenal hypoplasia congenita, hypogonadotropic hypogonadism, NR0B1 gene, DAX1, X-linked recessive

Abbreviations: ACTH, Adrenocorticotropic Hormone; AHC, Adrenal Hypoplasia Congenita; CAH, Congenital Adrenal Hyperplasia; DAX1, Dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X chromosome, gene 1; GnRH, Gonadotropin releasing Hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; VLCFA, very long chain fatty acid, 17-OH, hydroxyprogesterone; CT, computed tomography; HH, hypogonadotropic hypogonadism.

INTRODUCTION

The nuclear receptor subfamily 0 group B member 1 (*NR0B1)* gene encodes DAX1, an orphan nuclear receptor that plays a critical role in the development and differentiation of the adrenal gland and hypothalamus-pituitary-gonadal axis (1). It is predominantly expressed in the adrenal cortex, hypothalamus, pituitary gland, and gonads (testis and ovary). DAX1 has been proposed to serve as a transcription factor involved in the development of the adrenal cortex and pituitary gonadotropes (2). More than 200 mutations have been reported in *NR0B1*, most of which are nonsense or frameshift mutations that result in premature truncation of the protein (3). Defective *NR0B1* leads to X-linked adrenal hypoplasia congenita (AHC), resulting in a failure to develop the permanent adult adrenal cortex (4). Patients with *NR0B1* mutations may present with severe salt wasting in infancy or have a more insidious onset during childhood. AHC rarely presents with adrenal failure later in adulthood, and late-onset primary adrenal insufficiency (AI) is often accompanied by hypogonadotropic hypogonadism (HH) and azoospermia (5–7). In this study, we report a novel mutation in *NR0B1* that leads to late-onset AHC and failure of pubertal development.

MATERIALS AND METHODS

Subjects

The proband and all available family members were recruited from Wuhan Union Hospital (**Figure 1**). Medication history, disease symptoms, and progression of each family member was recorded by dictation as well as from medical records. Venous blood samples were collected from each family member after obtaining written informed consent. The present study was approved by the ethics committee of Wuhan Union Hospital.

DNA Sequencing

A library of genomic DNA was created from the proband's blood sample and enriched for exon targets according to the protocols of the Agilent SureSelect Human All Exon V6 Kit. After validation for size distribution using the Agilent 2100 Bioanalyzer, the enriched library was sequenced on the Illumina HiSeq X Ten platform. Raw files were generated and mapped to the human reference genome (GRCh37/hg19) using the Burrows-Wheeler alignment tool (version 0.7.8-r455). SAMtools was then applied to call single-nucleotide variants (SNVs) and indels (deletions and insertions, < 50 bp). Subsequently, ANNOVAR, accompanied by several prediction tools, was used to annotate SNVs and indels. Variants were annotated using genomic coordinates, referent nucleotides, variant nucleotides, mutation types, alleles, allele frequencies, gene names, amino acids variants and their evolutionary conservation. For allele frequency, each variant was compared against public population genetic databases. Based on the variant annotations, we focused on nonsynonymous coding substitutions, frameshifts, and splicing site mutations on chromosome X. Post these filtering steps, a list of candidate variants and related genes was created. To prioritize the most likely candidate disease-causing genes, all candidate genes were ranked using Phenolyzer (8). To validate the suspected variant in the proband, PCR and Sanger sequencing were performed on DNA samples of all available family members (II-2, II-3, II-6, III-1, III-2, III-3, III-4, and III-5) (**Figure 1**). The primer pairs used for PCR amplification of the region encompassing the suspected variant were forward 5′-GCTTTTAAAGAGCACCCGCC-3′ and reverse 5′-TTTCTTCACCTTTGCCCCGAC-3′.

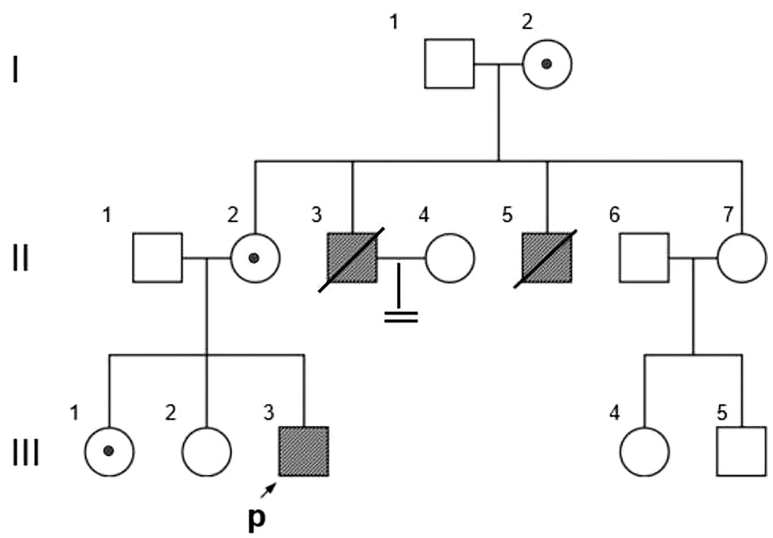

FIGURE 1 | Family Pedigree. Symbols represent males (squares), females (circles), affected subjects (solid symbols), carriers (dotted symbols), and the proband (arrow). The propositus is indicated by an arrow.

CASE PRESENTATION

Clinical Presentations

The pedigree in **Figure 1** shows the inheritance pattern of the X-linked recessive disorder. The proband (III-3), a 26-year-old male patient (height: 5 feet; weight: 75 kg; BMI 25.95), was admitted to the hospital with suspected adrenocortical failure. Medical history revealed that the patient had not exhibited secondary sexual characteristics by 17 years of age and had initial symptoms of skin hyperpigmentation; he had initiated prednisone oral tablet 5 mg per day along with some unknown Chinese herbs to help grow pubic hair. Up until the age of 24 years, there had been no incidence of fatigue, weight loss, dehydration, anorexia, nausea, or vomiting; however, in the following 2 years, he had developed worsening skin hyperpigmentation and experienced nausea and weakness following occasional treatment interruptions. Physical examination revealed generalized hyperpigmentation (**Figures 2A, B**) and delayed puberty, with the absence of secondary sexual characteristics (**Figures 2C–F**).

Laboratory examinations revealed a serum sodium level of 123.6 mmol/L at admission, which confirmed hyponatremia; however, it elevated to 136.6 mmol/L following hydrocortisone

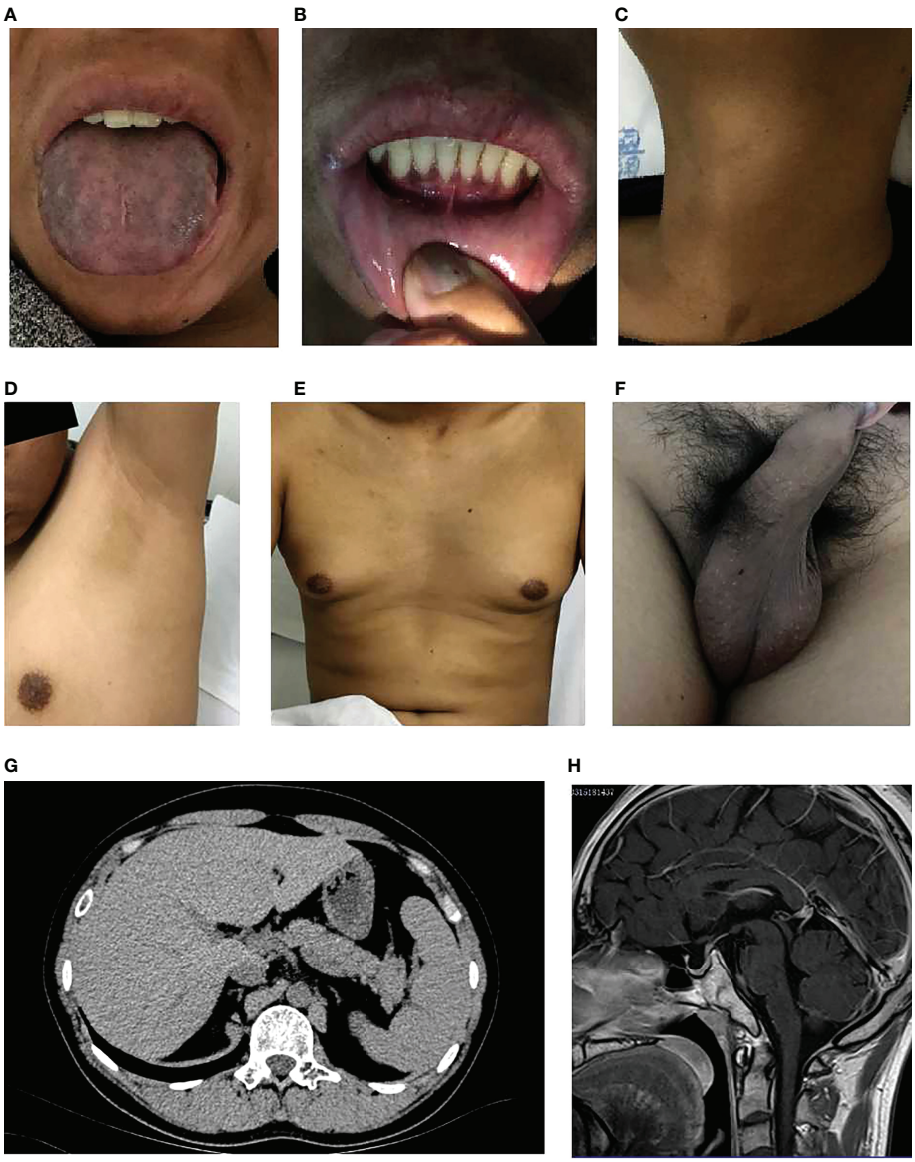

FIGURE 2 | Clinical characteristics of the proband. **(A)** Significant melanosis could be seen in the wrinkles of the face, gums, tongue, and limbs. **(B)** A few hyperpigmented macules were noted on the lips and oral mucosa. **(C-F)** Inconspicuous male secondary sexual characteristics: no beard, axillary hair, no obvious laryngeal knot, inverted triangle distributed pubic hair, significantly reduced bilateral testicles. **(G, H)**. Scanning results (adrenal hypoplasia, hypogonadotropic hypogonadism, small bilateral testes, olfactory sulci and pituitary, mild fatty liver).

treatment. The serum potassium levels were normal. The serum triglyceride level was extraordinarily high (29.57 mmol/L) at admission but dropped to 3.91 mmol/L following 5 days of lipid-lowering treatment. The serum cholesterol level was high (7.18 mmol/L). The plasma cortisol level (11.017 μg/L) was low. The adrenocorticotropic hormone level was extremely high, above the upper limit of detection (> 2000 pg/ml), which was consistent with primary AI. The VLCFA (very long-chain fatty acids) and 17-Hydroxyprogesterone concentrations were normal (**Table 1**). Initial hormone examinations showed normal levels of luteinizing hormone (2.49 IU/L) and follicle-stimulating hormone (3.08 IU/L) but a low level of testosterone (2.14 nmol/L). Thereafter, both gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin (hCG) stimulation tests were performed to diagnose the cause of hypotestosteronemia. As depicted in **Table 2**, in response to GnRH, testosterone levels increased from 1.96 IU/I to 10.27 IU/I at 90 s, but then decreased to 9.67 IU/I at 120 s. In response to hCG, testosterone levels increased by more than nine-fold, from 2.14 nmol/L to 18.33 nmol/L, after 48 h of stimulation, indicating that the testicular tissue was capable of achieving normal secretory function upon stimulation.

The scanning results provided additional evidence of adrenal hypoplasia and HH. Chromosomal analysis confirmed a normal male 46, XY karyotype. Bilateral testicular volume reduction was detected using B-ultrasound (left: 25.7*19.4*12.7 mm, right: 27.4*18.2*12.8 mm). Abdominal computed tomography revealed bilateral adrenal gland hypoplasia (**Figure 2G**). Magnetic resonance imaging of the olfactory sulci and pituitary gland was normal (**Figure 2H**). In addition, ultrasonography of the upper abdomen showed a mildly fatty liver.

Steroid supplementation with hydrocortisone (15-35 mg/d) and fludrocortisone (100 μg/d) resulted in a rapid improvement in the clinical conditions of the proband; the laboratory results following treatment are listed in **Table 1**. For personal reasons, in addition to androgen replacement, patient refused other treatments such as spermatogenesis therapy. During the follow-up, the patient refused to discuss his condition. His sibling mentioned that the proband's uncle died in a car accident, and the proband himself was bitter regarding not being able to have children, causing his depression and gloominess. He had abandoned his family plans.

Mutation Detection

Based on the reads aligned to the human reference genome (GRCh37/hg19), a total of 65,535 variants, including 58,704 SNVs and 6,831 indels, were identified. There were 965 variants located on chromosome X. Among them, 167 were nonsynonymous coding substitutions, and eight were frameshift variants. Prioritization with phenotype "adrenal hypoplasia" in Phenolyzer revealed a novel hemizygous frameshift deletion c.1034delC in the first exon of *NR0B1* at Xp21.2. A cytosine deletion at c.1034 caused a frameshift change, with proline-345 being the first affected amino acid by becoming arginine and forming a new reading frame resulting in a premature stop codon 27 amino acids further down (**Figure 3**). As a result, the putative ligand-binding domain (LBD) of the DAX protein was 20 amino acids shorter than the wild type. To validate the suspected variant in the proband, PCR and Sanger sequencing were performed on DNA samples of all available family members (II-2, II-3, II-6, III-1, III-2, III-3, III-4, and III-5), which revealed a hemizygous state in affected males (II-3 and III-3) and a heterozygous state in healthy female carriers (II-2 and III-1) (**Figure 1**).

DISCUSSION

X-linked AHC is an X-linked recessive inherited disease that typically affects males and presents as primary AI in early infancy or childhood, and HH at puberty with impaired spermatogenesis. Approximately 60% of male patients with AHC develop AI within 2 months after birth, whereas 40% do so between the age of 1 to 9 years. Recently, various cases of late-onset forms of X-linked AHC have been reported. In this study, we report a case of late-onset X-linked AHC and HH caused by a novel variant of *NROB1*. The proband presented with AI at 17 years of age and a lack of development of secondary sexual characteristics during puberty. Sequencing of *NR0B1* revealed a c.1034delC frameshift variant in two affected males in the kindred. The diagnosis of X-linked AHC and HH was established based on the clinical presentation, laboratory tests, and molecular analysis. According to the family history, two other male members of the family had presented with similar manifestations; one (II-3) was diagnosed with Addison's disease

TABLE 1 | Basal biochemical and hormone measurements.

Measures	At admission	After treatment	Normal value
Na/K	123.6/4.3	136.6/4.5	[135–150]/[3.5–5.5] Eq/L
ACTH	>2000	NA	[9–40] pg/mL
Cortisol	11.017	NA	[37–194] μg/L
Triglyceride	29.57	5.59	[<1.7] mmol/L
Cholesterol	7.18	5.28	[2.9-6.0] mmol/L
17-OHG	2.5	NA	[0.7–3.6ng/mL] ng/mL
VLCFA	0.30	NA	[<0.50] μg/ml
LH	2.49	NA	[1.7–8.6] IU/L
FSH	3.08	NA	[1.5-12.4] IU/L
Testosterone	2.14	NA	[8.6-29.0] nmol/L
Glucose	6.0	NA	[3.9-6.1] mmol/L

NA, not available.

TABLE 2 | GnRH stimulation test and hCG stimulation test.

GnRH	
Time	**LH(IU/L)**
0'	1.96
30'	6.85
60'	9.95
90'	10.27
120'	9.67
hCG	
Time	**Testosterone(nmol/L)**
0 hr	2.14
48 hr	18.33
72 hr	19.55

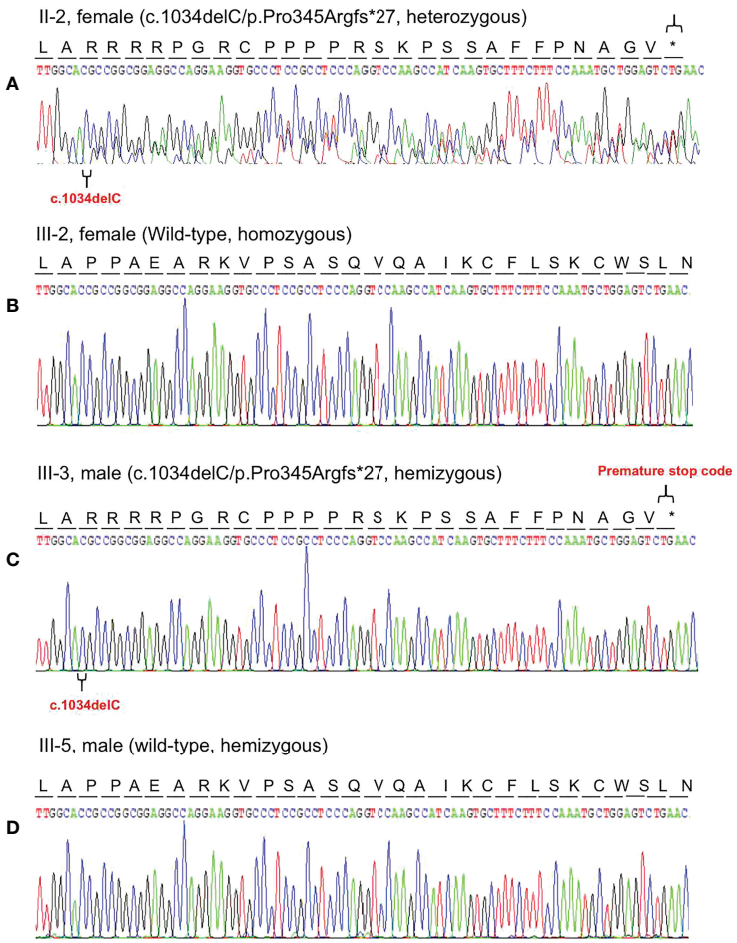

FIGURE 3 | Representative chromatogram of the *NR0B1* gene. The position of the mutation is indicated by special symbols ("Y" for the mutation, and "*" for Premature stop code). **(A)** The proband's mother (II-2) inherited the c.1034delC variant in heterozygous status. **(B)** His health sister (III-2) presented with homozygous wild-type of the *NR0B1* gene. **(C)** The proband was hemizygous for the c.1034delC variant in the *NROB1* gene. **(D)** His maternal cousin (III-5) showed hemizygosity for wild-type of the *NROB1*gene.

(AD) and male infertility at the age of 30 years and had been treated with prednisone, whereas the other (II-5), who had also been diagnosed with AD at the age of 33 years, succumbed to adrenal crisis at the age of 35 years. This indicates an X-linked mode of inheritance, as was confirmed by the results of the genetic analysis presented herein. In addition, no evident secondary causes of AD were identified, thus allowing us to exclude its presence.

NR0B1 consists of two exons and spans a 4.96 kb region at Xp21.2. It encodes a 470-amino-acid member of the nuclear hormone receptor superfamily. Most of the coding sequence is found in exon one (9, 10), which encodes the N-terminal domain and part of the C-terminal domain of the protein *NR0B1*. Exon two encodes the remaining part of the C-terminal domain. According to the statistical data of the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php, accessed on 20180630), a total of 252 mutations have been found in *NR0B1*. Truncating mutations such as nonsense or frameshift mutations have been identified throughout the gene. In contrast, missense changes have tended to cluster in the carboxyl terminus of the protein, corresponding to the putative LBD. Several cases of changes in the domain such as p.Ser259Pro, p.Pro279Leu, p.Ile439Ser, p.Tyr380Asp, and p.Gln305Hisfs*67 have been associated with a milder or late-onset phenotype (11). In functional studies of Tyr380Asp and Ile439Ser mutants, the partial loss of *NR0B1* transcriptional repression was consistent with the late-onset phenotype (12). The p.Pro345Argfs*27 mutation is a novel frameshift mutation within the LBD that introduces premature termination of the *NR0B1* protein, thus resulting in a truncated one. All the affected males in the family exhibited a late-onset phenotype. These findings suggest that the truncated *NR0B1* protein produced by this mutation may retain part of its function. Further studies are needed to elucidate the function of p.Pro345Argfs*27.

The same variant was identified in another male family member (II-5), who had presented with a dysfunction of the

adrenal cortex and had deceased owing to adrenal crisis. Although the dysfunction of his gonadal axis had occurred at an older age compared to that of the proband, it was more severe. His impairment involved the hypothalamus and pituitary, whereas the impairment of the proband primarily involved the hypothalamus. This suggests that, despite possessing the same genetic variant, there are differences in clinical manifestations, even in patients of the same pedigree. Liu also reported a nonsense variant change in exon 1 (c.192C>G) in two male siblings presenting with precocious puberty and late-onset HH but exhibiting different clinical manifestations (13).

There was no evident correlation between the *NR0B1* variant type and the clinical presentation. Literature search revealed 11 reports of adult-onset AHC (6, 7, 11, 14–20). The reported cases of adult-onset AI and hypogonadotrophic hypogonadism associated with mutations of *NR0B1* gene are summarized in **Supplementary Table S1**. Of the 11 reports, three (p.Gln37X in one, p.Trp39X in two kindreds) are amino-terminal nonsense mutations, whereas eight (p.Tyr378Cys, p.Leu386Phe, p.Ser259Pro in two kindreds, p.Pro279Leu, p.Gln305Hisfs*67 (c.915delG), p.Tyr380Asp, p.Ile439Ser) are mutations distributed throughout the carboxyl LBD. The mutation described in this study also falls in the "hotspot." Most patients presented with AI as the initial symptom followed by HH (or not); however, two cases presented with HH first, and further endocrine investigations confirmed AI. Among these variants, p.W39X was identified in two kindreds; the proband reported by M. Guclu et al. (17) presented with delayed puberty and small size of testes as the initial symptoms, whereas the other proband, reported by M. L. Raffin-Sanson et al., presented with fatigue, sore throat, and dizziness, which are consistent with AI (6). Moreover, the p.Ser259Pro mutation was also found in two families (11, 18), wherein both probands presented with AI initially, however, hypogonadism was absent in the case reported by C. M. Oh et al. (18). The reproductive phenotypes were variable in these patients, ranging from azoospermia to spontaneous fertility, even within the same family, as shown by M. C. C. Vargas et al. (20), referring to a family with spontaneous fertility and a variable spectrum of reproductive phenotypes, in which all males presented a distinct degree of testosterone deficiency and fertility phenotypes, ranging from a variable degree of hypogonadism, oligoasthenoteratozoospermia to spontaneous fertility. Additionally, the same variant can cause diverse phenotypes within one kindred as reported in a family with the p.Trp39X variant; the proband was diagnosed with AI at the age of 19 years but exhibited a preserved hypothalamic–pituitary–gonadal axis; in contrast, his nephew experienced AI crisis at the age of 2 weeks (6). The underlying mechanism remains obscure. The mutation by itself as well as the repressive role of *NR0B1* in steroidogenic factor-1 (SF1/Ad4BP, NR5A1) may be involved in this mechanism. DAX1 is expressed in tissues involved in steroid hormone production and reproductive function (21). This expression pattern overlaps significantly with that of another nuclear receptor, SF-1 (22). Previous studies have suggested that DAX1 is a negative regulator that represses SF-1-mediated transactivation of various genes involved in steroidogenesis (23). The loss of this inhibitory property in the LBD of DAX1 in the *NR0B1* variant has been demonstrated to be responsible for the pathology of X-linked AHC and HH (24–30).

In addition, the proband's serum triglyceride (TG) level was significantly high, which can be related to a low testosterone level. The plausible mechanism for the significant association between TG and testosterone levels in men is that low testosterone levels promote insulin resistance (31) by predisposing to visceral obesity, leading to a dysregulation of fatty acid metabolism, which in turn promotes insulin resistance (32). As reported previously, the relative risk of death in Swedish patients with AD is more than two-fold higher than that in the background population, primarily because of cardiovascular disease (CVD); elevated triglycerides are one of the CVD risk factors (33). Combining the dietary habits as well as no family history of hyperlipidemia makes it difficult to explain the common diseases of lipid metabolism. Therefore, whether or not the patient has a deletion of the glycerol kinase (GK) gene should be considered. The GK gene is located in Xp21.3, in a critical region of approximately 50–250 kb, close to the genomic coordinate of the AHC gene, and located distal to the Duchenne muscular dystrophy gene (34). Genomic analysis confirmed that the GK gene was unaffected, excluding combined glycerol kinase deficiency. Moreover, the serum VLCFA level was normal; therefore, adrenoleukodystrophy (ALD), another X-linked AI disorder, was also excluded. ALD is associated with primary testicular failure and characterized by primary AI and demyelination of the central or peripheral nervous system. The disease results from impaired transport of VLCFA into peroxisomes for beta-oxidation, which results in elevated levels of VLCFA in plasma and tissues (35).

CONCLUSION

In summary, we identified a novel frameshift variant of *NROB1*. This finding is important for expanding our knowledge of the phenotype and genotype of X-linked AHC. Functional studies of AHC patients are necessary to elucidate the biological effects of the identified mutation on the development and function of the adrenal gland and the hypothalamic–pituitary–gonadal axis.

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://ngdc.cncb.ac.cn/, HRA002312.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and

institutional requirements. Written informed consent was obtained from the patient prior to his inclusion in the study.

AUTHOR CONTRIBUTIONS

JX designed and led this study. FZ and MZ searched the literature and drafted the manuscript. XD collected patient information, recruited the patient. YL analyzed the data. JX revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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A maternal higher-complex carbohydrate diet increases bifidobacteria and alters early life acquisition of the infant microbiome in women with gestational diabetes mellitus

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Gestational diabetes mellitus (GDM) is associated with considerable imbalances in intestinal microbiota that may underlie pathological conditions in both mothers and infants. To more definitively identify these alterations, we evaluated the maternal and infant gut microbiota through the shotgun metagenomic analysis of a subset of stool specimens collected from a randomized, controlled trial in diet-controlled women with GDM. The women were fed either a CHOICE diet (60% complex carbohydrate/25% fat/15% protein, n=18) or a conventional diet (CONV, 40% complex carbohydrate/45% fat/15% protein, n=16) from 30 weeks' gestation through delivery. In contrast to other published studies, we designed the study to minimize the influence of other dietary sources by providing all meals, which were eucaloric and similar in fiber content. At 30 and 37 weeks' gestation, we collected maternal stool samples; performed the fasting measurements of glucose, glycerol, insulin, free fatty acids, and triglycerides; and administered an oral glucose tolerance test (OGTT) to measure glucose clearance and insulin response. Infant stool samples were collected at 2 weeks, 2 months, and 4–5 months of age. Maternal glucose was controlled to conventional targets in both diets, with no differences in Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). No differences in maternal alpha or beta diversity between the two diets from baseline to 37 weeks' gestation were observed. However, women on CHOICE diet had higher levels of *Bifidobacteriaceae*, specifically *Bifidobacterium adolescentis*, compared with women on CONV. Species-level taxa varied significantly with fasting glycerol, fasting glucose, and

glucose AUC after the OGTT challenge. Maternal diet significantly impacted the patterns of infant colonization over the first 4 months of life, with CHOICE infants showing increased microbiome alpha diversity (richness), greater *Clostridiaceae*, and decreased *Enterococcaceae* over time. Overall, these results suggest that an isocaloric GDM diet containing greater complex carbohydrates with reduced fat leads to an ostensibly beneficial effect on the maternal microbiome, improved infant gut microbiome diversity, and reduced opportunistic pathogens capable of playing a role in obesity and immune system development. These results highlight the critical role a maternal diet has in shaping the maternal and infant microbiome in women with GDM.

KEYWORDS

gut microbiota, pregnancy, infant, glycemic control, metagenomics, GDM

Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance that arises during pregnancy, has increased worldwide over the past decade, reaching up to 20% of all pregnancies depending on the diagnostic criteria (1). GDM is associated with increased risk for preeclampsia, cesarean section delivery, and preterm birth (2), with up to 50% of women developing type 2 diabetes within 10 years. Infants born to women with GDM have a higher risk of developing type 2 diabetes, obesity, and other chronic inflammatory diseases (3).

Diet is the primary treatment for GDM, and accordingly, the dynamic interactions between GDM and the maternal and infant gut microbiome have received substantial attention. The composition of the gut microbiota is altered in women with GDM during late pregnancy (4–6) and is associated in some cases with changes in blood glucose levels (7). Women who develop GDM undergo shifts in gut microbiota composition characterized by an enrichment of potential opportunistic pathogens in the family *Enterobacteriaceae*, while beneficial bacteria like *Bifidobacterium* or *Lachnospiraceae* are depleted (8). Higher abundances of *Enterobacteriaceae* have been linked to prediabetes and untreated type 2 diabetes (9) and increased circulating levels of lipopolysaccharide in adults, which can promote the development of obesity and insulin resistance by inducing a chronic inflammatory state (10). On the other hand, *Bifidobacterium* and *Lachnospiraceae* produce short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which help to decrease the production of pro-inflammatory cytokines and control the overgrowth of *Enterobacteriaceae* in adults (11).

Further, maternal and early life exposures during pregnancy and lactation, such as maternal obesity or diabetes, influence the development of the infant microbiome and have an impact on the occurrence of common diseases, such as diabetes, allergic and atopic disease, cardiovascular disease, and obesity in the offspring (12). Studies on the neonatal microbiome report lower richness and differences in microbial composition in infants born to women with GDM compared with women without a GDM diagnosis (13–16). However, to our knowledge, only one study has investigated the impact of GDM on the infant microbiome beyond 2 weeks of age (16) and no studies have investigated this paradigm between 2 weeks and 6 months of age. Evidence suggests that the microbiome within the first 2 months of life impacts immune cell development patterns up to 3 months of age (17), and others found an association between increased *Lachnospiraceae* abundance at 3–4 months of age and childhood overweight/obesity at 1 and 3 years (18). Therefore, a need exists for studies that investigate the microbiome of infants born to women with GDM within the first 4 months of life.

Although diet is a major driver of microbiota composition and functions (19), the impact of maternal diet therapy in women with GDM on the maternal and infant microbiome and potential consequences of diet therapy remains unexplored. Diet therapy for women with GDM conventionally involves lower carbohydrate intake to blunt postprandial glucose excursion, at the cost of increasing dietary fats (20) since protein intake is typically constant and difficult to appreciably modify. A maternal high-fat diet, especially with an increase in saturated fats, promotes insulin resistance in human and animal models (20, 21), can lead to increased fetal fat accretion (22, 23), and may put the offspring at a higher risk of developing metabolic syndrome due to excess lipid exposure *in utero* (24, 25). Thus, alternate diets are needed to improve maternal/infant health in women with GDM without the unintended consequences that may arise from administering a high-fat CONV diet. From a microbiome perspective, a need for more highly controlled diet studies (26) is warranted to investigate the effects of dietary intervention in women with GDM and how diet

impacts the maternal and infant microbiome and determine whether these alterations to the microbiome are linked to improvements in maternal and infant health outcomes. However, a previous study examining the maternal dietary effect on the maternal and infant microbiome in GDM had confounding influences of dietary intake that were not controlled (14). Here, we conducted a randomized, controlled dietary intervention (RCT) in GDM, directly comparing a conventional CONV diet or a diet higher in complex carbohydrates and lower in fat [CHOICE, Choosing Healthy Options in Carbohydrate Energy (27–29)]. Notably, all meals were provided for the duration of the study. The isocaloric nature of the diet was critical to ensure no differences in gestational weight gain (GWG) between diets. Furthermore, the percentage of saturated, polyunsaturated, and monounsaturated fats were identical and both simple sugars and fiber were also well controlled.

In the larger RCT of 46 women and infants who completed the study, we previously found that between-group maternal glycemic profiles were nearly identical (29). Although fasting free fatty acid (FFA) profiles were not different between the diet groups, the postprandial FFA profiles were lower with the CHOICE diet (29), likely suppressed by a higher postprandial insulin response. Between the diet groups, GWG was not different and insulin resistance indices were similar at 37 weeks, and the usual increase in the insulin resistance of pregnancy was blunted on both diets (29). Infant adiposity by air displacement plethysmography (PEAPOD), the primary endpoint on which the RCT was powered, was also not different, and cord blood glucose, C-peptide, FFAs, and triglycerides (TGs) were similar (29). From the larger RCT of 46 women, we collected a subset of stool samples in 34 women at 30 and 37 weeks' gestation and in 24 infants at 2 weeks, 2 months, and 4–5 months of age to examine the impact of a controlled, third-trimester diet on the maternal microbiome and on infant microbial colonization during the first 4 months of life. We hypothesized that the consumption of the CHOICE, compared with CONV, would favorably alter maternal and infant microbiome composition consistent with improvements to the maternal measures of glucose tolerance.

Materials and methods

Participants

This study was approved by the Colorado Multiple Internal Review Board and was registered at http://www.clinicaltrials.gov (NCT02244814). All studies were performed in accordance with relevant guidelines and regulations. GDM was diagnosed using the Carpenter and Coustan criteria (30) between gestational weeks 24 and 28. Women were randomized to a diet group and entry criteria included the age of 20–36 years, a BMI of 26–39 kg/m^2, a singleton pregnancy, no significant or obstetric comorbidities, no history of preterm labor or preeclampsia prior to term, and the treatment of GDM with diet alone. Informed consent was obtained from all women, and all women planned to breastfeed for at least 4 months and were otherwise healthy. Participants were excluded if they met any criteria for overt diabetes or were likely to fail the diet and require medical attention (fasting glucose >115 mg/dl or fasting TGs >400 mg/dl, which would place them at risk for TG-induced pancreatitis). Women taking beta blockers, antihypertensives, or glucocorticoids were excluded as were smokers and non-English speaking women. Maternal stool samples were excluded if they took antibiotics within 4 weeks of the sampling time. Infant stool samples were excluded if their mother received antibiotics at delivery, if they took antibiotics within 4 weeks of the stool sample collection, or if they were missing information on antibiotic use at the sample collection. From the original cohort of 46 women with GDM, 34 women in this subgroup had complete measurement data and stool samples from the study visits at 30 and 37 weeks' gestation (n=16, CONV; n=18, CHOICE). Twenty-four infants had stool samples collected (n=14, CONV; n=10, CHOICE).

Study protocol

The enrolled women with newly diagnosed GDM began dietary intervention between gestational weeks 30 and 31 and continued to delivery. Detailed information on the diets has been described (28). Briefly, diets were eucaloric, contained similar amounts of fiber (~23.5 and ~29.3 g/day for CONV and CHOICE, respectively), and had the following macronutrient distributions: CONV, 40% complex carbohydrate/45% fat/15% protein; CHOICE, 60% complex carbohydrate/25% fat/15% protein. Both diets were matched for fat percentage (35% saturated fatty acids/45% monounsaturated fatty acids/20% polyunsaturated fatty acids) and simple sugars (≤18% of kilocalories) and were composed of foods with a low-to-moderate glycemic index. Daily kilocalories were distributed as 25% breakfast/25% lunch/30% dinner/20% snacks. We defined complex carbohydrates as "polysaccharides and starches primarily derived from grains, vegetables, and fruits that tend to attenuate a sharp postprandial rise in plasma glucose" (28). All menus were tailored to individual participant food preferences, and meals were prepared by the Clinical Translational Research Center Nutrition Services at University of Colorado Anschutz Medical Campus. Meals were picked up by the participants or delivered every 72 h when they met with investigators. Women were provided with formula to supplement or replace breastfeeding when necessary (Gerber Good Start, Arlington, VA, USA). Supplementary Figure 1 shows a summary of the study design.

Blood measures

Maternal blood measures were performed at two different visits at 30–31 and 36–37 weeks' gestation. A fasting (10-h) blood sample was collected prior to a 2-h oral glucose tolerance test (OGTT) at baseline (30–31 weeks) and again after 6–7 weeks on the diet (36–37 weeks). OGTT measurements were taken at 0, 30, 60, 90, and 120 min using a peripheral intravenous line. The Matsuda index was calculated using the standard method (31). HOMA-IR was calculated as [fasting insulin x (fasting glucose/18)]/22.5. Hepatic insulin resistance was calculated by multiplying glucose AUC by insulin AUC calculated during the first 30 min of the OGTT. GWG was determined by the change in weight from the first prenatal visit to delivery and weight gain on diet by the change in weight from time of diet randomization to delivery. Additional blood measures (i.e., TGs, FFAs, glycerol, glucose, C-peptide, and insulin) were performed at breakfast meal studies at baseline and 36–37 weeks' gestation, where participants consumed a standardized breakfast meal (30% of total daily energy intake) after an overnight fast (≥10 h) as previously (28). When possible, cord blood was obtained to measure infant C-peptide, glucose, insulin, and HOMA-IR.

Infant measurements

The infant breastfeeding status was determined by a questionnaire and was grouped by whether they were exclusively breastfed (yes, no, or mixed). The delivery mode was recorded as vaginal, cesarean section with labor, or cesarean section without labor; however, we grouped the cesarean section variables together due to low sample size. Birth weight and infant anthropometrics were obtained and newborn percent body fat was measured by air displacement plethysmography (PEAPOD) at delivery and the 2-week, 2-month, and 4–5 month postpartum visits (COSMED, Rome, Italy) (28).

Stool sample collection and metagenomics processing

Maternal stool samples were collected at 30–31 and 36–37 weeks' gestation by mothers within 24 h of their clinic visit and stored at -20°C until delivery to the laboratory, whereupon aliquots were separated and stored at -80°C until analysis. Infant stool samples were obtained at 2 weeks, 2 months, and 4–5 months, and DNA extraction was carried out as previously described using the QIAamp PowerFecal DNA kit (Qiagen Inc, Carlsbad, CA, USA). Triplicate shotgun metagenomic libraries were constructed for each stool sample (except for one pair of maternal samples, which was sequenced in duplicate) using the plexWell LP384 kit (seqWell Inc., Beverly, MA, USA) following the manufacturer's protocol. The shotgun metagenomic sequencing of the pooled libraries was performed on the NovaSeq 6000 (Illumina, San Diego, CA, USA) at 2 × 150-bp read length by Novogene Inc. (Sacramento, CA, USA). Raw sequence reads were trimmed and processed for quality using BBMap (32). Briefly, the default options in bbduk were used to remove the adapter sequence and quality trimming before removing contaminant sequences with the Kmer filtering option. The tadpole command was then used in the error correction mode using default parameters. The triplicate/duplicate runs were then concatenated for further processing. The average sequencing depth, average number of contigs, and average contig length are shown in Supplementary Table 1. Metaphlan2 was used to retrieve the taxonomy and relative abundances using default settings (33). The participants' sequence libraries at all timepoints were further concatenated for contig assembly using MEGAHIT (34). Contigs ≥1,000 bp were mapped to the MEGAHIT coassembly using bowtie2 (35). Contigs were predicted for gene function with Prodigal (36) using default settings and annotated to the Kyoto Encyclopedia of Genes (KEGG) database (37–39) using KofamScan (40). Reads per kilobyte million (RPKMs) were calculated for each annotation for downstream analysis.

Statistical analysis

Population characteristics

Maternal BMI and blood measures were log-scaled then standardized using the mean and standard deviation; the normality of the data was checked using a Shapiro–Wilk test. To test for differences between the groups, a repeated-measures ANOVA was performed on the continuous variables against the diet group and time. Continuous infant characteristics were modeled using the same repeated-measures ANOVA procedure as for the maternal characteristics, and categorical variables were tested for differences between the diet groups using a chi-squared test.

Microbial analysis

Bacterial sequence counts were rarefied to 100,000 reads per sample, without replacement, 999 times and averaged across all sampling iterations before rounding to the nearest integer. Rarefaction curves were run to confirm that sample richness reached an asymptote within 100,000 reads and Good's coverage was used to confirm adequate rarefaction (>99% for each sample) (41). Shannon diversity and Chao1 richness indices, calculated at the family, species, and gene annotation levels, were used as the measures of alpha diversity, and the Bray–Curtis dissimilarity index was used as a measure of beta diversity. Alpha and beta diversities were calculated using the vegan package (42).

Modeling

Models were constructed at the family level of taxonomy using alpha diversity, beta diversity, and taxonomic abundances—a deeper insight into species-level differences in taxonomic abundances was also calculated. Gene annotation abundances were investigated using the same modeling procedures. To evaluate these models, we used the function lmer from the lme4 package (43) for alpha diversity and the gene annotation data (linear mixed effects models), adonis2 from the vegan package for beta diversity (PERMANOVA),and glmmer.nb from the lme4 package for taxonomic abundance comparisons (negative binomial regression). For each dataset, modeling was performed as follows: 1) our simplest model included the diet group, sampling timepoint, and the interaction of diet group and sample timepoint within the framework of a repeated-measures design. 2) Additional models were built by adding a single explanatory variable to the simple model (12 additional models in the maternal analysis and four in the infant analysis). 3) A full model was constructed by adding all variables into one multivariate regression. The corrected Akaike information criterion (AICc) was computed for each of the models, where a lower AICc is indicative of a better model fit and an AICc score of 2 or lower suggests a significantly better model fit (44, 45). 4) If multiple explanatory variables from (2) had AICc scores 2 or lower than the simple model, the variables were added to a model together with group and time, and the AICc was calculated once more. 5) If there was a large difference between the AICc scores used in (4), the variable from (2) within the best-fitting model (i.e., the one with the lowest AICc) and variables in models within 2 AICc scores of the best-fitting model were included together in an additional model and the AICc again calculated. Once computed, the AICc scores were compared and the lowest AICc model was used for statistical analysis. If there were multiple models within 2 AICc points of each other, the simpler model of the two was used (e.g., if a model from (2) was close in AICc to our simple model (1), we would choose the simple model). If both models are equally parsimonious, the lower AICc model was chosen. If a model failed to converge or was found to be singular between the taxon and the variable of interest, the model was discarded.

Once the final models were calculated, the P-values were compiled and corrected for the false discovery rate (FDR) using the Benjamini–Hochberg procedure. AICc was calculated using the MuMIn package (46), except for the adonis2 models, which used a custom R script to calculate AICc from the residual sums of squares (47). In the negative binomial regression models, taxa at the family and species levels were included in the modeling if they were present in at least 60% of the samples and had at least 5,000 reads summed across all samples. The number of OTUS and percent read coverage after applying these filtering procedures are shown in Supplementary Table 1. To define the gene annotation based on mapping genes in the KEGG Orthology database, we used KEGG orthologous terms present in at least 60% of the samples, where they were then summed into gene families for further analysis using lmer.

Results

Maternal and infant characteristics were similar between the diet groups

Similar to the findings in the whole cohort (29), we found no differences in maternal characteristics between the diet groups in this subgroup (Table 1). The majority of infants in this subgroup were delivered vaginally (~70%) and exclusively breastfed throughout the collection period, and the body fat percentage was not different between the diet groups (29) (Table 2).

Maternal microbial and gene-annotation diversity did not differ between the diet groups and were not associated with any maternal measures

We first evaluated whether the overall structure of the maternal microbiome was altered by the dietary intervention. To this end, we assessed the significance of alpha- and beta-diversity measures from maternal stool samples at the family, species, and gene annotation levels. At the family level, both alpha diversity metrics—Shannon diversity and Chao1 (richness)—were best modeled by the simplest model, which included only the diet group, time, and their interaction. However, none of these variables were significantly associated with either alpha diversity metric ($p>0.05$) (Supplementary Figure 2). At the species level, Shannon diversity was best modeled by the simplest model, but no terms were significant ($p>0.05$) (Supplementary Figure 3). Chao1 was best modeled by the full model, which included the diet group, time, and their interaction as well as all 11 of the maternal measurements. However, none of these variables were significantly associated with Chao1 richness at the species level ($p>0.05$). At the gene annotation level, we found results similar to those of the microbiome at the species level, where Shannon diversity was best modeled by the simplest model, but no terms were significant ($p>0.05$), and Chao1 was best modeled by the full model, but no terms were significant ($p>0.05$) (Supplementary Figure 4).

In the family level, beta-diversity models, the simplest model fit the data best, but the diet group, time, and their interaction were not associated with maternal microbiome beta diversity ($p>0.05$) (Supplementary Figure 5). Similar results were found at the species (simplest model, all $p>0.05$) and gene annotation levels (full model, all $p>0.05$) (Supplementary Figures 6 and 7).

Maternal microbiome displayed moderate associations with diet and maternal measures at the family level

Next, we identified individual taxa with differential abundances in maternal microbiota at the family level and found several taxa associated with maternal measures (Table 3

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TABLE 1 Maternal population characteristics.

Group	CONV		CHOICE		p-value
Maternal age (years)[1]	32.96 ± 3.08		31.96 ± 4.77		0.49
Days on diet[1]	33.75 ± 4.17		33.0 ± 6.12		0.68
Weight gain on diet (kg)[1]	0.5 ± 1.43		0.62 ± 1.29		0.81
GWG (kg)[1]	8.9 ± 5.79		10.27 ± 4.47		0.44
Ethnicity, n (%)					0.21
Caucasian	11 (68.8)		16 (88.9)		
Asian	4 (26.7)		1 (5.6)		
American Indian or Alaska Native	1 (6.3)		0 (0)		
Other	0 (0)		1 (5.6)		
Parity, n (%)					0.19
0	6 (37.5)		12 (66.7)		
1	9 (56.3)		4 (22.2)		
2	1 (6.3)		1 (5.6)		
3	0 (0)		1 (5.6)		
Timepoint	**30 Weeks**	**37 Weeks**	**30 Weeks**	**37 Weeks**	
Sample Size, n	16	16	18	18	
Maternal BMI (kg/m^2)[1]	31.47 ± 4.95	31.71 ± 4.86	32.93 ± 5.69	33.16 ± 5.79	0.94
Fasting Glucose (mg/dl)[1]	77.81 ± 7.17	73.56 ± 6.53	79.83 ± 6.49	72.72 ± 6.14	0.23
Fasting Insulin (uIU/ml)[1]	11.5 ± 5.89	11.62 ± 5.29	15.39 ± 6.57	13.67 ± 4.59	0.25
log2(Glucose AUC)[1]	14.19 ± 0.16	14.17 ± 0.17	14.14 ± 0.11	14.05 ± 0.14	0.11
log2(Insulin AUC)[1]	13.3 ± 0.9	13.44 ± 0.78	13.54 ± 0.65	13.64 ± 0.6	0.85
Fasting Free Fatty Acids[1]	8.79 ± 0.42	8.83 ± 0.32	8.85 ± 0.44	8.84 ± 0.43	0.74
Fasting Triglycerides[1]	198 ± 60.05	226.25 ± 56.43	216.78 ± 54.26	260.61 ± 58.89	0.27
Fasting Glycerol[1]	100.44 ± 34.78	102.38 ± 36.65	113.17 ± 29.48	126.44 ± 41	0.45
Hepatic IR[1]	22.07 ± 0.89	21.93 ± 0.78	22.27 ± 0.72	22.16 ± 0.62	0.84
HOMA IR[1]	2.23 ± 1.22	2.12 ± 1	3.08 ± 1.43	2.47 ± 0.9	0.16
Matsuda Index[1]	3.48 ± 1.89	3.4 ± 1.48	2.57 ± 0.87	2.77 ± 0.85	0.44

[1]Values shown are mean ± SD.
GWG, gestational weight gain.

and Supplementary Figure 8). We found that *Rikenellaceae* was negatively associated with maternal fasting FFA levels (p=0.02). Other associations were found between *Bifidobacteriaceae* and insulin AUC and an increase in *Bifidobacteriaceae* abundance over time on CHOICE but a decrease on CONV. However, these associations did not survive FDR correction. Likewise, we found that *Prevotellaceae* abundance was positively associated with the Matsuda index and *Erysipelotrichaceae* was similarly associated with HOMA-IR, but these associations did not survive FDR correction. A full list of models and their respective results are available in Supplementary Table 2.

CHOICE diet increased maternal abundance of *Bifidobacterium adolescentis*

At the species level, we found that several taxa were associated with maternal diet and other maternal measures (Table 4 and Supplementary Figure 9). *Bifidobacterium adolescentis* was the main species responsible for the *Bifidobacteriaceae* increase on CHOICE and decrease on CONV (p=0.02). Similarly, *Prevotella copri* showed a trend decrease on CHOICE but increase on CONV (p=0.07). Two species of *Alistipes* were differentially associated with fasting glucose levels, with *A. finegoldii* displaying a negative relationship (p=0.04) while *A. shahii* had a positive relationship (p=0.03). *A. shaii*, *A. finegoldii*, *Alistipes putredinis*, and *Bacteroides vulgatus* were all negatively associated with fasting glycerol levels, but the association with *A. shahii* did not survive FDR correction. Four species, *Streptococcus thermophilus, Eubacterium ramulus, Dorea longicatena*, and *Roseburia hominis* were associated with glucose AUC after the OGTT challenge. However, only the association with *E. ramulus* was significant (p<0.001), while the others showed trend associations after FDR correction (p<0.1). A full list of models and their respective results are available in Supplementary Table 3.

Maternal gene annotation pathways were not altered by diet

Finally, we analyzed maternal microbiome gene annotations and found several associations between maternal measures and

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TABLE 2 Infant population characteristics.

Group		CONV			CHOICE		p- value
Sample Size, n		14			10		
Vaginal Delivery, n (%)		11 (78.57)			6 (60.0)		0.59
Female, n (%)		7 (50.0)			6 (60.0)		0.94
Gestation Period (months)[1]		39.4 ± 0.3			38.9 ± 0.35		0.31
Birth Weight (g)[1]		3254 ± 101			3083 ± 119		0.29
% Body Fat at Delivery[1]		7.7 ± 7.5			7.5 ± 1.3		0.94
Cord C-peptide[1,a]		0.78 ± 0.23			0.86 ± 0.44		0.65
Cord Fasting Glucose[1,b]		58.25 ± 8.31			63.25 ± 11.32		0.40
Cord Fasting Insulin[1,c]		4.00 ± 1.00			3.75 ± 1.26		0.72
Ethnicity, n (%)							0.48
White		11 (78.6)			7 (70.0)		
Asian		3 (21.4)			2 (20.0)		
American Indian or Alaska Native		0 (0)			1 (10.0)		
Infant Age	**2 Weeks**	**2 Months**	**4–5 Months**	**2 Weeks**	**2 Months**	**4–5 Months**	
Sample Size, n	11	13	11	9	10	9	
Infant Weight (kg)	3.50 ± 0.36	5.24 ± 0.71	6.60 ± 0.78	3.23 ± 0.41	4.96 ± 0.51	6.32 ± 0.60	0.21
% Body Fat	10.39 ± 4.7	21.93 ± 4.0	25.19 ± 4.3	8.43 ± 4.3	18.37 ± 5.8	23.53 ± 3.9	0.24
Exclusively Breastfed, n (%)							0.71
No	1 (9.09)	1 (7.69)	2 (18.18)	0 (0)	1 (10.0)	2 (22.22)	
Mixed	3 (27.27)	3 (23.08)	2 (18.18)	0 (0)	1 (10.0)	1 (11.11)	
Yes	7 (63.64)	9 (69.23)	7 (63.64)	9 (100)	8 (80.0)	6 (66.67)	

[1]Values shown are mean ± SD.
[a]Missing 10 observations; CONV n=9, CHOICE n=5.
[b]Missing 12 observations; CONV n=8, CHOICE n=4.
[c]Missing 13 observations; CONV n=7, CHOICE n=4.

metabolic, transport, degradation, and infection-related pathways, but none of them survived FDR correction. A full list of models and their respective results are available in Supplementary Table 4.

Microbial family-level richness increased with age in infants born to women on CHOICE

Next, we evaluated the overall structure of the infant microbiome and whether it was altered by maternal dietary intervention. We assessed the significance of alpha- and beta-diversity measures in the infant stool samples at the family, species, and gene annotation levels. At the family level, we found no association between Shannon diversity and diet group, infant age, or the interaction of the two terms (Figure 1A). The infant microbiome Chao1 index decreased over time in CONV infants and increased in CHOICE infants (p=0.009), resulting in a significantly higher richness score in the CHOICE infants compared with CONV at 4–5 months (Figure 1B). Cesarean section delivery was associated with increased microbiome richness compared with infants born by vaginal delivery (Figure 1C). At the species level, Shannon diversity was associated with a trend increase over time (p=0.06), while the Chao1 index was not associated with any terms (p>0.05), as best modeled by the full model (diet, age, diet/age interaction and GWG, delivery mode, breastfeeding status, and sex) (Supplementary Figure 10). At the gene annotation level, results were similar to those found at the species level, and no terms were significant for the simplest model in Shannon diversity (p>0.05) or for the full model in the Chao1 index (p>0.05) (Supplementary Figure 11).

Bray–Curtis beta diversity at the family level was modeled best by the simple model and displayed a trend association with the diet group (p=0.06) and diet group by infant age (0.09) (Supplementary Figure 12). At the species level, we found a significant difference in the infant gut community between the diet groups (p=0.01) and how the microbiota developed over time (p=0.01) (Supplementary Figure 13) but not their interaction (p=0.18). Similar results were found at the gene annotation level but only reached trend significance (diet group and age, p=0.06, Supplementary Figure 14; diet and age interaction p=0.10).

Infant family-level abundances were altered with infant age, maternal diet group, and their interaction

Like we did with the maternal microbiome data, we identified individual taxa with differential abundances in the infant microbiota at the family level. Multiple taxa differed in

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TABLE 3 Variables associated with maternal family-level taxa determined by negative binomial regression.

Variable/Taxa					p-value	Adjusted p-value
Timepoint[1]	30 weeks	37 weeks				
Bifidobacteriaceae	1.91 ± 0.38	2.04 ± 0.4			0.03	0.34
Diet Group by Timepoint[1]	CONV 30W	CONV 37W	CHOICE 30W	CHOICE 37W		
Bifidobacteriaceae	2.07 ± 0.59	1.46 ± 0.44	1.76 ± 0.48	2.85 ± 0.73	0.004	0.12
Matsuda Index[2]	Predicted Slope					
Prevotellaceae	0.47 ± 0.27				0.01	0.19
log2(Fasting FFAs)[2]						
Rikenellaceae	-0.002 ± 0.001				<0.001	0.02
HOMA-IR[2]						
Erysipelotrichaceae	0.22 ± 0.21				0.02	0.24
log2(Insulin AUC)[3]						
Bifidobacteriaceae	0.22 ± 0.21				0.01	0.19

[1]Values shown are mean ± SEM.
[2]Number refers to the change in relative abundance per 1 unit increase in the variable being reported.
[3]Number refers to the change in relative abundance per 1 unit increase in the log-adjusted variable being reported.

abundance in association with maternal or infant variables (Table 5 and Supplementary Figure 15). *Staphylococcaceae* and *Streptococcaceae* were enriched at 2 weeks of age and decreased in abundance over time at 2 months and 4–5 months of age (both p<0.001). *Ruminococcaceae* increased at 2 months and remained stable through 4–5 months of age (p<0.001), while *Veillonellaceae* also increased in abundance at 2 months but decreased at 4–5 months of age (p<0.001). *Coriobacteriaceae* and *Eubacteriaceae* increased in abundance over time (p=0.03 and p<0.001, respectively). Two families were associated with the diet group alone: CONV infants had higher levels of *Enterococcaceae* (p=0.002) and a lower abundance of *Clostridiaceae* (p=0.03).

Several families were significantly associated with the diet group and infant age. *Clostridiaceae* began higher in CHOICE at 2 weeks of age and decreased at 2 months but remained stable in the CONV infants (p<0.001). *Eubacteriaceae* was stable in CHOICE infants but was enriched at 4–5 months in CONV infants (p<0.001) Finally, *Veillonellaceae* began at a high abundance in 2-week-old CONV infants and decreased over time, while this family was low in abundance in CHOICE infants at 2 weeks of age and increased in abundance over time (p=0.04).

Independent of the infant age, *Lactobacillaceae* was impacted by the delivery mode; infants delivered vaginally had a lower abundance than infants born *via* cesarean section (p=0.01). The abundance of *Verrucomicrobiaceae* was higher in female infants (p<0.001). *Enterococcaceae* and *Lachnospiraceae* were both positively associated with maternal GWG, where *Enterococcaceae* increased by 0.18% relative abundance for every 1 kg gained during pregnancy (p=0.04) and *Lachnospiraceae* increased by 0.48% relative abundance for every kilogram (p=0.006). A full list of models and their respective results are available in Supplementary Table 5.

Species-level abundances in infants change over time and displayed different colonization patterns between maternal diet groups

At the species level, *B. breve*, *B. longum*, *Collinsella aerofaciens*, *B. ovatus*, *B. thetaiotamicron*, *E. rectale*, *Faecalibacterium prausnitzii*, and *Subdoligranulum* unclassified increased in abundance from 2 weeks to 4–5 months (Table 6 and Supplementary Figure 16). The opposite association was observed for *Staphylococcus epidermidis*, *S. anginosus*, *S. vestibularis*, *Clostridium perfringens*, *Veillonella dispar*, and *Haemophilus parainfluenza*, which decreased in abundance as the infant aged. *V. atypica* increased in abundance at 2 months of age before returning to the level similar to that seen at 2 weeks. Four species were associated with diet—*Enterococcus faecalis*, *S. anginosus*, *C. perfringens*, and *V. parvula*—all of which were higher in CONV infants independent of infant age.

Several species were associated with changes in abundance within a diet group over time. *B. ovatus*, *S. anginosus*, and *Enterobacter cloacae* displayed a pattern of abundance in CONV infants that changed over time (either up or down), whereas no change in the abundance of these species was observed in CHOICE infants. With *C. perfringens*, increased abundance was seen at 2 weeks of age in CHOICE infants and decreased by 2 months, whereas this species was depleted in CONV infants at 2 weeks and increased in abundance at 2 months. Similar differential colonization patterns over time between the diet groups were also seen for *B. longum*, *V. parvula*, and *Veillonella* unclassified.

C. perfringens was higher in abundance in infants born *via* cesarean section. *S. epidermidis* and *S. anginosus* were both higher in abundance in exclusively breastfed infants compared

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TABLE 4 Variables associated with maternal species–level taxa determined by negative binomial regression.

Variable/Taxa					p-value	Adjusted p-value
Timepoint[1]	30 weeks	37 weeks				
Bifidobacterium adolescentis	0.58 ± 0.2	0.43 ± 0.16			0.01	0.12
Eubacterium eligens	0.33 ± 0.11	0.41 ± 0.12			0.04	0.22
Anaerostipes hadrus	0.14 ± 0.07	0.26 ± 0.09			0.05	0.24
Dorea longicatena	0.73 ± 0.17	0.75 ± 0.17			0.02	0.15
Coprobacillus unclassified	0.06 ± 0.04	0.12 ± 0.06			0.04	0.22
Diet Group[1]	CONV	CHOICE				
Bifidobacterium longum	1.07 ± 0.23	0.86 ± 0.2			0.03	0.19
Bacteroides xylanisolvens	0.3 ± 0.1	0.16 ± 0.07			0.03	0.19
Clostridium symbiosum	0.14 ± 0.08	0.03 ± 0.03			0.03	0.19
Eubacterium hallii	1.04 ± 0.22	2.52 ± 0.41			0.01	0.08
Ruminococcus bromii	0.17 ± 0.12	0.88 ± 0.49			0.02	0.15
Klebsiella oxytoca	0.03 ± 0.03	0.11 ± 0.06			0.03	0.20
Diet Group by Timepoint[1]	CONV 30W	CONV 37W	CHOICE 30W	CHOICE 37W		
Bifidobacterium adolescentis	0.54 ± 0.28^{ab}	0.16 ± 0.1^{b}	0.62 ± 0.28^{ab}	1.17 ± 0.5^{a}	0.001	0.02
Prevotella copri	0.19 ± 0.19	0.36 ± 0.35	0.14 ± 0.14	0.07 ± 0.08	0.01	0.07
Eubacterium ramulus	0.14 ± 0.09	0.18 ± 0.1	0.33 ± 0.14	0.24 ± 0.13	0.02	0.14
Lachnospiraceae bacterium	0.05 ± 0.06	0.03 ± 0.04	0.07 ± 0.06	0.13 ± 0.09	0.04	0.22
Matsuda index[2]	Predicted Slope					
Bifidobacterium bifidum	-0.57 ± 0.34				0.01	0.12
Parabacteroides distasonis	0.42 ± 0.23				0.01	0.09
Maternal BMI[2]						
Bacteroides fragilis	-0.15 ± 0.06				0.04	0.22
Bacteroides thetaiotaomicron	-0.1 ± 0.05				0.03	0.19
Alistipes finegoldii	-0.078 ± 0.065				0.02	0.15
HOMA-IR[2]						
Bacteroides uniformis	0.38 ± 0.16				0.004	0.07
Fasting Glucose[2]						
Alistipes finegoldii	-0.049 ± 0.044				0.002	0.04
Alistipes shahii	0.033 ± 0.032				0.001	0.03
Anaerostipes hadrus	0.04 ± 0.04				0.02	0.15
Ruminococcus bromii	-0.07 ± 0.03				0.005	0.07
log2(Fasting Triglycerides)[3]						
Faecalibacterium prausnitzii	-1.35 ± 0.29				0.01	0.12
log2(Fasting FFAs)[3]						
Ruminococcus obeum	-0.29 ± 0.35				0.02	0.13
Escherichia coli	0.83 ± 0.43				0.02	0.14
Escherichia unclassified	1.78 ± 0.83				0.01	0.08
log2(Fasting Glycerol)[3]						
Bacteroides thetaiotaomicron	-0.21 ± 0.24				0.01	0.12
Bacteroides vulgatus	-0.91 ± 0.28				<0.001	0.01
Alistipes finegoldii	-0.53 ± 0.52				<0.001	0.01
Alistipes putredinis	-0.53 ± 0.52				<0.001	0.01
Alistipes shahii	-0.38 ± 0.59				0.03	0.19
log2(Insulin AUC)[3]						
Bacteroides fragilis	-0.32 ± 0.35				0.01	0.11
Eubacterium ventriosum	1.2 ± 0.37				0.01	0.13
log2(Glucose AUC)[3]						

(Continued)

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TABLE 4 Continued

Variable/Taxa		p-value	Adjusted p-value
Streptococcus thermophilus	-3.33 ± 1.95	0.005	0.07
Eubacterium ramulus	3.56 ± 1.67	<0.001	<0.001
Dorea longicatena	1.67 ± 1.15	0.003	0.06
Roseburia hominis	-3.73 ± 1.4	0.003	0.06

Values in a row that do not contain the same superscript are significantly different, p<0.05
[1]Values shown are mean ± SEM.
[2]Number refers to the change in relative abundance per 1 unit increase in the variable being reported.
[3]Number refers to the change in relative abundance per 1 unit increase in the log-adjusted variable being reported.

with mixed feeding and formula-only feeding. Female infants had a trend for higher abundance of *S. anginosus* compared with male infants. Three species were positively associated with GWG: *E. faecalis* increased by 0.14% relative abundance per 1 kg of weight gained, *S. salivarius* by 0.42%, and *R. gnavus* by 0.01%. A full list of models and their respective results are available in Supplementary Table 6.

Infant gene annotation pathways were not different between maternal diet groups

Finally, we analyzed infant microbiome gene annotations and found only one pathway that was significant with any of the tested maternal/infant variables after FDR correction. A trend between the mannose-type O-glycan biosynthesis pathway and the breastfeeding status was observed (p=0.09) where infants who

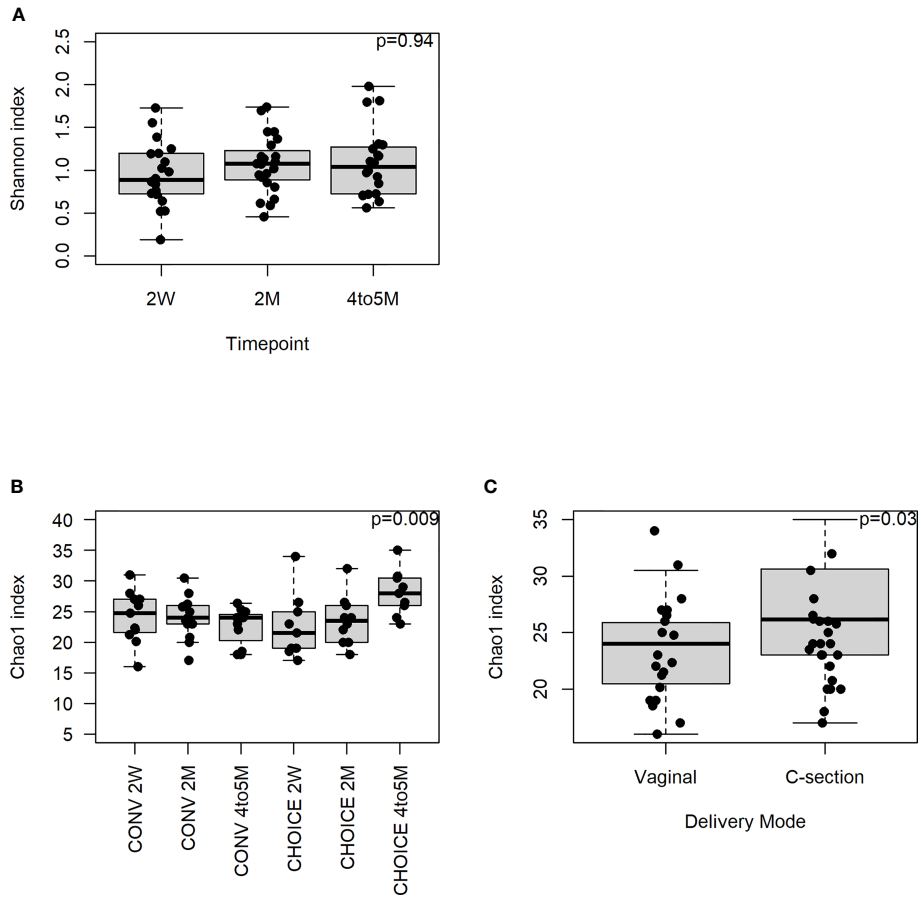

FIGURE 1
Infant microbiome alpha diversity at the family level. A repeated-measures mixed-effects model was used to test for statistical significance. **(A)** Association of Shannon diversity and infant age (n=20, 2-week; n=23, 2-month; n=20, 4–5-month). **(B)** Chao1 index association with diet group over time (n=11, CONV 2-week; n=13, CONV 2-month; n=11, CONV 4–5-month; n=9, CHOICE 2-week; n=10, CHOICE 2-month; n=9, CHOICE 4–5-month). **(C)** Association of Chao1 index with delivery mode (n=17, vaginal; n=7, cesarean).

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TABLE 5 Variables associated with infant family-level taxa determined by negative binomial regression.

Variable/Taxa							p-value	Adjusted p-value
Infant Age	2 Weeks	2 Months	4-5 Months					
Coriobacteriaceae	0.02 ± 0.01^{c}	0.13 ± 0.06^{b}	0.51 ± 0.27^{a}				0.005	0.03
Staphylococcaceae	0.91 ± 0.42^{a}	0.05 ± 0.02^{b}	0.01 ± 0.01^{c}				<0.001	<0.001
Streptococcaceae	6.35 ± 1.98^{a}	1.16 ± 0.34^{b}	0.87 ± 0.27^{b}				<0.001	<0.001
Clostridiaceae	0.54 ± 0.22	0.18 ± 0.07	0.3 ± 0.12				0.04	0.15
Eubacteriaceae	0.01 ± 0.002^{b}	0.01 ± 0.003^{b}	0.15 ± 0.04^{a}				<0.001	<0.001
Ruminococcaceae	0.04 ± 0.02^{b}	0.15 ± 0.06^{a}	0.2 ± 0.09^{a}				<0.001	<0.001
Verrucomicrobiaceae	0.01 ± 0.004^{c}	0.42 ± 0.14^{a}	0.05 ± 0.02^{b}				<0.001	<0.001
Diet Group	CONV	CHOICE						
Enterococcaceae	0.43 ± 0.13	0.07 ± 0.03					<0.001	0.002
Clostridiaceae	0.29 ± 0.16	0.4 ± 0.25					0.01	0.03
Ruminococcaceae	0.17 ± 0.08	0.06 ± 0.04					0.05	0.16
Veillonellaceae	4.49 ± 2.32	1.55 ± 0.94					0.03	0.12
Diet Group by Infant Age	CONV 2W	CONV 2M	CONV 4-5M	CHOICE 2W	CHOICE 2M	CHOICE 4-5M		
Clostridiaceae	0.11 ± 0.07^{ab}	0.45 ± 0.29^{ab}	0.5 ± 0.34^{ab}	1.84 ± 1.41^{a}	0.09 ± 0.07^{b}	0.39 ± 0.3^{ab}	<0.001	<0.001
Eubacteriaceae	0.01 ± 0.003^{b}	0.01 ± 0.003^{b}	0.88 ± 0.34^{a}	0.01 ± 0.003^{b}	0.01 ± 0.01^{b}	0.02 ± 0.01^{b}	<0.001	<0.001
Veillonellaceae	6.59 ± 3.96^{a}	5.37 ± 3.1^{a}	2.56 ± 1.54^{ab}	0.93 ± 0.64^{b}	1.23 ± 0.83^{ab}	3.24 ± 2.23^{ab}	0.01	0.04
Delivery Mode	Vaginal	C-section						
Lactobacillaceae	0.12 ± 0.05	0.97 ± 0.53					0.002	0.01
Eubacteriaceae	0.02 ± 0.01	0.01 ± 0.01					0.03	0.12
Sex	Male	Female						
Verrucomicrobiaceae	0.02 ± 0.01	0.15 ± 0.04					<0.001	<0.001
GWG[1]	Predicted Slope							
Enterococcaceae	0.18 ± 0.04						0.01	0.04
Lachnospiraceae	0.48 ± 0.22						0.001	0.01

Values in a row that do not contain the same superscript are significantly different, $p<0.05$
All values shown are mean ± SEM.
[1]Number refers to the increase in relative abundance per 1 kg increase in gestational weight gain (GWG).

were not breastfed had a much higher abundance of genes involved in this pathway than mixed feeding or exclusive breastfeeding (Supplementary Figure 17). A full list of models and their respective results are available in Supplementary Table 7.

Discussion

To our knowledge, this is the first study to compare maternal and infant gut microbiome outcomes in a dietary intervention of two different diet compositions (all meals provided) in women with GDM. We ascertained how the diets impacted the maternal gut microbiome and the relationships with maternal metabolic characteristics and the infant microbiome under eucaloric diet conditions and equivalent GWG. Surprisingly, we found that the probiotic family *Bifidobacteriaceae*, specifically *B. adolescentis*, increased in the microbiota of women on the CHOICE diet. *Bifidobacteria* are generally beneficial bacteria that attenuate intestinal inflammation and dysbiosis, in part by enhancing SCFA production (7), inhibiting and reducing lipopolysaccharide-induced injury of the gut epithelium (48), and by metabolizing resistant starches such as human milk oligosaccharides and other complex carbohydrates like fructooligosaccharides and galactooligosaccharides (49). *B. adolescentis* abundance is correlated with lower HbA1c and basal insulin requirements (50), suggesting that it conveys an overall protective effect of the CHOICE diet on pancreatic β-cell function in women with GDM. Others have found similar increases in *Bifidobacteria* after supplementing adults with prediabetes with galactooligosaccharides for 12 weeks (51). Furthermore, other studies suggest that the supplementation of resistant starches in healthy adults also increases *Bifidobacteria* (52), while the depletion of dietary carbohydrates in a gluten-free diet results in a likewise depletion of *Bifidobacteria* (53). Together, this suggests that higher intake of complex carbohydrates in the diet, in pregnant and non-pregnant individuals, increases *Bifidobacteriaceae* abundance in the microbiome.

The maternal microbiome at the family and species levels also varied significantly with the fasting levels of FFAs, glycerol, and glucose AUC after the OGTT challenge. Specifically, we found a negative association between *Rikenellaceae* and fasting FFAs. Other studies in adults have found an inverse association

TABLE 6 Variables associated with infant species–level taxa determined by negative binomial regression.

Variable/Taxa							p value	Adjusted p value
Infant Age	2 Weeks	2 Months	4-5 Months					
Bifidobacterium adolescentis	0.02 ± 0.01	0.04 ± 0.03	0.03 ± 0.02				0.036	0.151
Bifidobacterium bifidum	0.03 ± 0.01	0.03 ± 0.02	0.08 ± 0.04				0.028	0.125
Bifidobacterium breve	0.82 ± 0.46^{b}	2.94 ± 1.62^{a}	4.22 ± 2.37^{a}				0.002	0.017
Bifidobacterium longum	0.55 ± 0.28^{b}	1.58 ± 0.78^{b}	8.02 ± 4.14^{a}				0.002	0.017
Collinsella aerofaciens	0.002 ± 0.001^{b}	0.003 ± 0.002^{b}	0.02 ± 0.02^{a}				0.001	0.011
Bacteroides ovatus	0.01 ± 0.01^{b}	0.02 ± 0.01^{b}	0.04 ± 0.02^{a}				<0.001	<0.001
Bacteroides thetaiotaomicron	0.006 ± 0.003^{b}	0.007 ± 0.004^{b}	0.01 ± 0.008^{a}				0.003	0.025
Staphylococcus epidermidis	0.15 ± 0.06^{a}	0.002 ± 0.001^{b}	0.001 ± 0.0005^{b}				<0.001	<0.001
Streptococcus anginosus	0.015 ± 0.007^{a}	0.003 ± 0.001^{b}	0.001 ± 0.0005^{b}				<0.001	<0.001
Streptococcus vestibularis	1.79 ± 0.88^{a}	0.17 ± 0.08^{b}	0.02 ± 0.01^{c}				<0.001	0.002
Clostridium perfringens	1.0 ± 0.47^{a}	0.21 ± 0.10^{b}	0.11 ± 0.05^{b}				0.016	0.075
Eubacterium rectale	0.008 ± 0.002^{b}	0.01 ± 0.003^{ab}	0.015 ± 0.005^{a}				0.014	0.069
Faecalibacterium prausnitzii	0.007 ± 0.002^{b}	0.009 ± 0.003^{ab}	0.01 ± 0.004^{a}				0.009	0.052
Subdoligranulum unclassified	0.02 ± 0.01^{b}	0.05 ± 0.02^{a}	0.09 ± 0.04^{a}				<0.001	0.002
Veillonella atypica	0.02 ± 0.01^{b}	0.05 ± 0.02^{a}	0.03 ± 0.01^{ab}				0.010	0.058
Veillonella dispar	0.04 ± 0.02^{a}	0.03 ± 0.02^{ab}	0.01 ± 0.01^{b}				0.012	0.062
Enterobacter cloacae	0.03 ± 0.02	0.06 ± 0.03	0.03 ± 0.02				0.032	0.138
Haemophilus parainfluenzae	0.006 ± 0.006^{ab}	0.009 ± 0.009^{a}	0.002 ± 0.002^{b}				0.017	0.078
Diet Group	CONV	CHOICE						
Bifidobacterium longum	2.26 ± 1.27	1.61 ± 1.06					0.045	0.177
Enterococcus faecalis	0.35 ± 0.11	0.05 ± 0.02					<0.001	0.001
Streptococcus anginosus	0.02 ± 0.008	0.0006 ± 0.0003					<0.001	<0.001
Clostridium perfringens	0.41 ± 0.15	0.2 ± 0.08					<0.001	<0.001
Veillonella parvula	1.06 ± 0.59	0.15 ± 0.1					<0.001	0.003
Diet Group by Infant Age	CONV 2W	CONV 2M	CONV 4-5M	CHOICE 2W	CHOICE 2M	CHOICE 4-5M		
Bifidobacterium longum	1.55 ± 1.06^{ab}	0.94 ± 0.61^{b}	7.89 ± 5.36^{a}	0.19 ± 0.15^{c}	2.63 ± 1.99^{ab}	8.15 ± 6.34^{ab}	0.003	0.022
Bacteroides ovatus	0.01 ± 0.01^{b}	0.01 ± 0.01^{b}	0.09 ± 0.06^{a}	0.02 ± 0.02^{ab}	0.02 ± 0.02^{ab}	0.02 ± 0.02^{ab}	0.004	0.028
Enterococcus faecalis	0.77 ± 0.42	0.27 ± 0.14	0.22 ± 0.12	0.03 ± 0.02	0.03 ± 0.02	0.15 ± 0.09	0.043	0.175
Streptococcus anginosus	0.57 ± 0.31^{a}	0.01 ± 0.007^{b}	0.001 ± 0.001^{c}	0.0004 ± 0.0003^{c}	0.0005 ± 0.0003^{c}	0.001 ± 0.0007^{c}	<0.001	<0.001
Clostridium perfringens	0.13 ± 0.08^{c}	1.43 ± 0.84^{ab}	0.36 ± 0.24^{b}	7.78 ± 5.32^{a}	0.03 ± 0.02^{c}	0.03 ± 0.02^{c}	<0.001	<0.001
Veillonella parvula	3.35 ± 2.8^{a}	0.9 ± 0.64^{ab}	0.4 ± 0.3^{ab}	0.05 ± 0.04^{b}	0.18 ± 0.15^{ab}	0.36 ± 0.28^{ab}	0.006	0.035
Veillonella unclassified	0.39 ± 0.36^{ab}	0.49 ± 0.44^{ab}	0.13 ± 0.12^{b}	0.3 ± 0.32^{ab}	0.34 ± 0.36^{ab}	1.24 ± 1.31^{a}	0.006	0.035
Enterobacter cloacae	0.05 ± 0.04^{a}	0.05 ± 0.04^{a}	0.01 ± 0.01^{a}	0.02 ± 0.02^{ab}	0.06 ± 0.06^{a}	0.08 ± 0.07^{a}	0.012	0.062
Klebsiella unclassified	0.01 ± 0.01	0.04 ± 0.04	0.03 ± 0.03	0.09 ± 0.1	0.02 ± 0.02	0.05 ± 0.06	0.027	0.124
Delivery Mode	Vaginal	C-section						
Clostridium perfringens	0.05 ± 0.01	1.82 ± 0.84					<0.001	<0.001
Sex	Male	Female						
Streptococcus anginosus	0.002 ± 0.0008	0.006 ± 0.003					0.012	0.062
Breastfeeding Status	No	Mixed	Exclusive					
Staphylococcus epidermidis	0.002 ± 0.002^{b}	0.004 ± 0.002^{b}	0.05 ± 0.01^{a}				<0.001	<0.001
Streptococcus anginosus	0.003 ± 0.002^{b}	0.0007 ± 0.0004^{b}	0.02 ± 0.005^{a}				<0.001	<0.001

(Continued)

TABLE 6 Continued

Variable/Taxa		p value	Adjusted p value
GWG[1]	Predicted Slope		
Enterococcus faecalis	0.14 ± 0.03	<0.001	0.001
Streptococcus salivarius	0.42 ± 0.08	0.005	0.030
Ruminococcus gnavus	0.01 ± 0.003	<0.001	0.002

Values in a row that do not contain the same superscript are significantly different, p<0.05.
All values shown are mean ± SEM.
[1]Number refers to the increase in relative abundance per 1 kg increase in gestational weight gain (GWG).

between this family and the levels of visceral adipose tissue in elderly adults (54), a negative association with obesity (55), and depleted abundance in adults with non-alcoholic fatty liver disease (56). Interestingly, species within the genus *Alistipes*, part of the family *Rikenellaceae*, were inversely associated with fasting glycerol and fasting glucose levels but not FFAs. *A. finegoldii* and *A. putredinis* were negatively associated with fasting glycerol levels along with *B. vulgatus*. Blood glycerol levels are a measure of adipose tissue lipolysis and TGs (57) and feed directly into the gluconeogenesis pathway. Together, our data suggest that bacteria in the family *Rikenellaceae* help lower FFAs, but specific members of the family (i.e., *Alisitpes*) have a relationship with a different part of the same pathway, notably fasting glycerol and glucose levels.

B. uniformis was positively associated with HOMA-IR in our cohort, although this was only a trend association after FDR correction. Supplementation studies have found that *B. uniformis* can reduce body weight gain, liver TGs, and inflammation in the context of a high-fat diet in mice (58, 59). However, in humans with metabolic syndrome, increases in the stool levels of *B. uniformis* had no effect on body composition or insulin sensitivity (60). Pregnant women with type 1 diabetes have increased abundance of *B. uniformis* (61). More work needs to be done to elucidate the role of *B. uniformis* in diabetes risk.

Maternal diet significantly impacted the patterns of infant colonization over time, with CHOICE infants showing increased microbiome alpha diversity (richness), greater *Clostridiaceae*, and decreased *Enterococcaceae*. To our knowledge, there are only two studies that have investigated the impact of maternal diet on the infant microbiome in humans. Lundgren et al. (62) observed that increased maternal fruit and vegetable intake was associated with higher levels of *Streptococcus* and *Clostridium* and decreased abundances of *Enterobacteriaceae* in offspring at 6 weeks of age. Chu et al. (19) found that a maternal diet higher in fat was associated with greater *Enterococcus* abundance in the meconium and higher *Bacteroides* abundance in infants at 6 weeks of age. We found a similar result in our cohort, where *Enterococcaceae* abundance was increased in infants born to mothers on the higher-fat CONV diet. *Enterococcaceae*, like *E. faecalis*, is known to influence healthy intestinal immune system development, but the members of this family can also act as opportunistic pathogens (63). In healthy infants, this family decreased in abundance as other bacteria began to colonize and populate the gut, suggesting that early *Enterococcaceae* colonization is important for proper immune training but an overabundance in the early life could increase the risk of *Enterococcus* infection.

The abundances of infant microbiome species were impacted by the maternal diet group as the infant aged. For example, *C. perfringens* was enriched in the gut of CONV infants; a relative abundance of 1.43% and 0.36% was observed at 2 months and 4–5 months of age, respectively. In contrast, *C. perfringens* started at a higher abundance at 2 weeks of age in CHOICE infants before dropping to 0.03% relative abundance at 2 and 4–5 months. Other groups have associated cesarean section delivery with a higher abundance of *C. perfringens* (64), which is consistent with our results. Moreover, the microbiome of germ-free mice colonized with gut microbes from infants born to women with obesity was enriched in *Clostridia* (65) and the enrichment of this taxa has been associated with adolescent obesity (66). This suggests that the higher-fat CONV diet exacerbated obesity-associated microbiome signals within a population that is already at risk for adverse health events due to the maternal GDM status. We found an association between a higher abundance of *Lactobacillaceae* and cesarean section delivery, which is surprising considering that vaginally delivered infants have a much higher exposure to vaginal *Lactobacillus* species compared with cesarean section–delivered infants. However, a recent systematic review by Shaterian et al. concluded that cesarean section delivery is associated with a higher abundance of *Lactobacillus* in the first 3 months of life, while vaginally delivered infants have higher abundances of this taxa after 3 months of age (67), which matches our results. Moreover, gut *Lactobacillus* species do not appear to originate from the maternal vaginal microbiome (68), likely due to differences in species-specific adaptations needed to colonize the vagina versus the human gastrointestinal tract. Together, this suggests that the delivery mode impacts *Lactobacillaceae* colonization not because of the direct transmission of *Lactobacillus* from a mother to a child but because of differential successional patterns of colonization prompted by early bacterial exposures. In our other comparisons, we the family *Lachnospiraceae* was

positively associated with maternal GWG and was predicted to increase in relative abundance by 0.5% for each kilogram increase in GWG. Other studies have noted a positive association between maternal overweight/obesity and infant *Lachnospiraceae* abundance (13, 18), which was linked to infant risk for overweight/obesity later in life (18). More studies are needed to determine whether individual genera/species within *Lachnospiraceae* are contributing to infant adiposity and whether specific metabolites produced by this family are responsible for this association with overweight/obesity risk in offspring.

Two species in infant stool were associated with the breastfeeding status: *S. epidermidis* and *S. anginosus*, both of which were enriched in the gut of infants who were exclusively breastfed. *S. epidermidis* is acquired from skin and can be found in breastmilk, may play a role in educating the immune system and can act as an opportunistic pathogen in neonates (69). Less is known about the role of *S. anginosus* in the commensal human gut, although it has been shown to be a common member of the skin, respiratory, and intestinal microbiomes and has the potential to cause infection (70). Breastfeeding for the first 6 months of life is the recommended practice to optimally support infant survival, nutrition, and development (71). Our results suggest that exclusive breastfeeding results in distinct communities in the infant gut and increases the abundance of bacteria important for immune training.

A major strength of our RCT study is that the mothers were well matched, the study design allowed for well-controlled dietary constituents that impact the microbiome (fiber, calories, all meals provided), and GWG was nearly identical. We also controlled for antibiotic exposure, excluding any maternal or infant stool sample when antibiotics were reported within 4 weeks of collection. Importantly, we collected maternal and infant stool samples at several timepoints, allowing us to interrogate how the maternal diet group related to changes in the microbiome across gestation and infant age. Another strength is that the gut microbiota of study participants was not confounded by anti-hyperglycemic agents. Our trial also had certain limitations, including the small sample size and the predominantly Caucasian participants, and thus needs confirmation in a larger RCT including women with different ethnicities. Additionally, due to limitations inherent in macronutrient research, we cannot be certain that the differences reported in women on CHOICE are due to a higher intake of carbohydrates rather than a lower intake of fats. However, we emphasize carbohydrate intake in this study since the administration of complex carbohydrates to women with GDM makes this study unique and provides an alternative to the higher-fat CONV GDM diet. Although the infant sample size was small, all results were corrected for FDR, and subspecies occurrence with divergent functionalities was identified with metagenomic sequencing. Many of the bacteria investigated were, on average, less than a percent of the total relative abundance in the participants' microbiome. While the higher-abundance taxa will interact more with the host and gut environment, rare taxa have been shown to disproportionately contribute to shifts in a microbiome community over time (72) and may act as metabolic keystones to bridge gaps between the functional repertoires of the microbiome as a whole (73). The entire study cohort was powered to detect differences in postprandial FFA AUC, placental FATP2 expression, and neonatal adiposity (primary outcome) between groups. However, this subanalysis of the study trial may not be adequately powered to detect small differences in microbiota abundances and should thus be interpreted with caution. As this was an exploratory study, more work needs to be done in a larger cohort. In addition, although the two groups were extremely well matched for age, BMI, parity, and ethnic distribution (see Table 1), we cannot exclude other socioeconomic factors or unknown environment differences in the two cohorts. The goal of these studies was to recruit, to the greatest extent possible, women with similar starting metabolic characteristics, the ability to follow their diets (compliance), and without the need to go on to require insulin therapy. More work needs to be done to elucidate the roles of rare taxa in the maintenance of a healthy microbiome and the functional pathways in a larger cohort, despite their relative rarity.

Overall, our results show that an isocaloric diet high in complex carbohydrates and low in fat consumed by women with GDM is associated with a bacterial environment that is metabolically favorable, demonstrating greater bacterial diversity and a reduction in potential pathogens in infants during the first 4 months of life. Improving the gut microbiome diversity and reducing opportunistic pathogens capable of playing a role in obesity and immune system dysbiosis suggest the exciting possibility of using a targeted intervention to modulate these taxa and modify disease progression in a precision medicine–based approach in future studies. The potential implications of the abundance of these taxa for overall metabolic health across various phenotypes of infants from women with GDM await further elucidation.

Data availability statement

The shotgun metagenomic data generated and analyzed during the current study are deposited in the NCBI Sequence Read Archive under BioProject ID PRJNA845806. https://www.ncbi.nlm.nih.gov/sra.

Ethics statement

The studies involving human participants were reviewed and approved by Colorado Multiple Internal Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TH and LB designed the clinical study from which samples were generated. DF and JF provided guidance on the study design related to microbiome study data interpretation and analysis. JK processed stool samples for sequence acquisition. KS performed biostatistical and bioinformatic analyses. All authors contributed to and approved the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.921464/full#supplementary-material

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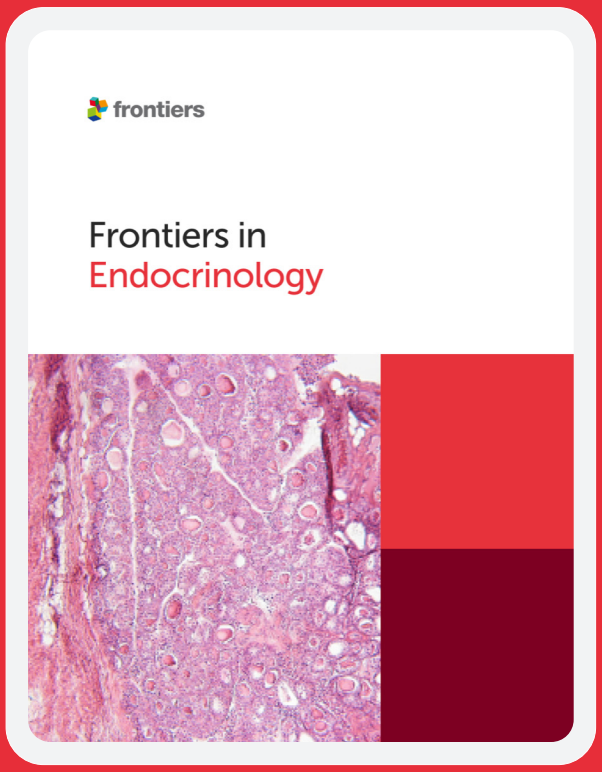

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