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Original Article

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Rodents on a high-fat diet born to mothers with gestational diabetes exhibit sex-specific lipidomic changes in reproductive organs

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Abstract

Maternal gestatonal diabetes mellitus (GDM) and offspring high-fat diet (HFD) have been shown to have sex-specific detrimental effects on the health of the offspring. Maternal GDM combined with an offspring HFD alters the lipidomic profiles of offspring reproductive organs with sex hormones and increases insulin signaling, resulting in offspring obesity and diabetes. The pre-pregnancy maternal GDM mice model is established by feeding maternal C57BL/6 mice and their offspring are fed with either a HFD or a low-fat diet (LFD). Testis, ovary and liver are collected from offspring at 20 weeks of age. The lipidomic profiles of the testis and ovary are characterized using gas chromatography-mass spectrometry. Male offspring following a HFD have elevated body weight. In reproductive organs and hormones, male offspring from GDM mothers have decreased testes weights and testosterone levels, while female offspring from GDM mothers show increased ovary weights and estrogen levels. Maternal GDM aggravates the effects of an offspring HFD in male offspring on the AKT pathway, while increasing the risk of developing inflammation when expose to a HFD in female offspring liver. Testes are prone to the effect of maternal GDM, whereas ovarian metabolite profiles are upregulated in maternal GDM and downregulated in offspring following an HFD. Maternal GDM and an offspring HFD have different metabolic effects on offspring reproductive organs, and PUFAs may protect against detrimental outcomes in the offspring, such as obesity and diabetes.

Key words maternal GDM, mice offspring, metabolomics, hormone, insulin signaling

Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance with onset or first recognition during pregnancy [1], influences about 18.4 million pregnancies worldwide annually [2,3]. In China, the incidence of GDM is approximately 17.5% [4]. Maternal GDM is associated with short-term and long-term adverse health outcomes in their offspring later in life [5]. These offspring are at risk of poor metabolic health, including impaired glucose tolerance, impaired insulin secretion, changes in adipokines [6,7], and increased risk of diabetes and obesity throughout childhood and adulthood [8,9].

High-fat diet (HFD) is a major contributor to chronic metabolic diseases and obesity worldwide [10,11]. Previous rodent studies have demonstrated that a maternal HFD could lead to sex-specific responses in their offspring, with female offspring having increased lipid, glucose and insulin levels in the serum [12], whilst male offspring exhibit detrimental effects on elevated fasting serum levels

of free fatty acids [13]. It is widely acknowledged that sex steroid hormones contribute to sex-specific differences in body composition. Feeding offspring with an HFD postnatally has been found to cause alterations in metabolic and hormonal profiles, such as elevated levels of glucose, insulin, and lower testosterone levels [14,15].

Previous studies have elucidated that maternal obesity combined with an offspring high carbohydrate diet results in dynamic alterations of the lipidomic profiles of adipose tissue in male offspring [16,17]. Although some studies have directly investigated the effects of HFD consumption in the offspring after exposure to GDM in utero [18,19], few studies have investigated its effect on metabolic health outcomes with a focus on sex-specific effects. The effects of an offspring diet intervention and maternal GDM on insulin signaling pathways and sex hormones in reproductive organs of the offspring remains unclear.

In this study, we aim to evaluate sex-specific lipidomic changes in reproductive organs and hormones in the offspring after exposure to maternal GDM in utero combined with an offspring HFD.

Materials and Methods

HFD-induced GDM mouse model

All wild-type C57BL/6 mice in this study were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All animal experiments complied with the ARRIVE guidelines and approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Batch number: 2020-41).

The mice were randomly divided into two groups ($n = 20$ per group): control or GDM group after adaptive feeding for one week. The normal maternal mice were fed with a low-fat diet consisting of 20.3% protein, 63.9% carbohydrate, and 15.8% fat (Research Diets AIN-93G) from weaning. A high-fat diet (HFD) consisting of 20% protein, 35% carbohydrate, and 45% fat (Research Diets D12451) was utilized for one week prior to mating and throughout pregnancy (18.5 days) to build a GDM mouse model that closely resembled metabolic abnormalities similar to human GDM [20]. Female mice in the normal and GDM groups were mated with males of the same genotype in a ratio of 1:2 at week 12. The overall experimental design is illustrated in Figure 1A. The protein was obtained from casein, isolated soybean protein, egg white solids, lactalbumin and wheat gluten. The carbohydrate was from sucrose and cornstarch. The fat source was the soybean oil [21].

After 16.5 days, OGTT was performed by first fasting mice for six hours and then administering the mice with glucose (2 g/kg body weight) via gavage. At 0, 30, 60, 90, and 120 min, blood samples were collected from the tail vein, and a glucometer (Nova StatStrip Xpress; Nova Biomedical, Waltham, UK) was used to measure the blood glucose concentration. After 18.5 days, maternal blood samples were collected from tail vein after six hours of fasting. The serum was collected and separated by centrifugation for 10 min at 4000 *g* and 4°C, and the insulin levels were measured using the ELISA kit (Beyotime, Shanghai, China).

Offspring generation and diet intervention

The GDM mice were mated with the C57BL/6 male mice and the offspring were reduced to seven pups after birth to avert food competition during the suckling period. All parental mice were continuously fed with the low-fat diet (LFD). Supplementary Table S1 showed the birth information of offspring from the normal and GDM maternal mice. All the offspring were separated from the maternal mice from three weeks old. Mice from the same litter were separated into different cages depending on their allocation and fed with an LFD. At 8 weeks of age, mice were fed with either an LFD or an HFD. This led to the establishment of eight diverse experimental groups: Female offspring from GDM mothers who were then fed with an LFD (F-G-L, $n = 8$); female offspring from GDM mothers who were then fed with an HFD (F-G-H, $n = 6$); female offspring from normal mothers who were then fed with an LFD (F-N-L, $n = 9$); female offspring from normal mothers who were then fed with an HFD (F-N-H, $n = 10$); male offspring from GDM mothers who were then fed with an LFD (M-G-L, $n = 8$); male offspring from GDM mothers who were then fed with an HFD (M-G-H, $n = 9$); male offspring from normal mothers who were then fed with an LFD (M-N-L, $n = 6$); and male offspring from normal mothers who were then fed with an HFD (M-N-H, $n = 7$).

Measurement of offspring characteristics

Offspring body weights were measured at 8, 14 and 20 weeks of age. At 20 weeks of age, the systolic blood pressure was recorded using the method with "tail-cuff" by a blood pressure recorder (IITC Life Science, Woodland Hills, USA). The mice tails were occluded with the proper size tube-shaped tail cuff linked to the tail cuff device and the basal level blood pressure was recorded according to the instruction [22,23]. The OGTT was performed in offspring at 20 weeks of age and the protocol was identical to that in the maternal mice.

At 20 weeks of age, blood samples were only collected from a tail vein after 6 h of fasting, and the collected serum was separated by centrifugation for 10 min at 4000 *g* and 4°C, and frozen at −80°C for storage. The concentrations of plasma insulin, estrogen, and testosterone were determined using the corresponding ELISA kits (Beyotime) and the concentrations of plasma FSH were measured using the ELISA kit obtained from Jianglai Biotechnology (Shanghai, China) according to the manufacturer's instructions.

At 20 weeks of age, the offspring livers were collected after 6 h of fasting for the Folch lipid extraction which was performed for the isolation in offspring liver and purification of total lipids from offspring liver following the previous protocol [24].

Western blot analysis

Proteins were extracted from the mice liver, gonadal adipose tissue, ovaries and testes with RIPA lysis buffer (Thermo Scientific, Waltham, USA). Protein concentrations were measured using a BCA estimation kit (Thermo Scientific) according to the manufacturer's protocol. Western blot analysis was performed following the instruction. Protein samples were subject to SDS-PAGE (7%, 10% or 12%) and then transferred to PVDF membranes (Millipore, Billerica, USA). The Primary antibodies were anti-ERS1 (1:1000 dilution; Abcam, Cambridge, UK), anti-AR (1:1000 dilution; Abcam), anti-IRS1 (1:1000 dilution; Abcam), anti-pIRS1 (1:1000 dilution; Abcam), anti-PI3K (1:1000 dilution; Abcam), anti-pPI3K (1:1000 dilution; Abcam), anti-AKT (1:1000 dilution; Abcam), anti-pAKT (1:1000 dilution; Abcam), anti-TNFα (1:1000 dilution; Abcam), and β-actin (1:1000 dilution; Abcam). The secondary antibody was goat-anti mouse IgG and goat-anti rabbit IgG (1:5000 dilution; Abcam). The protein bands were scanned and relative intensity of each band was quantified using Quantity One software (Bio-Rad, Hercules, USA).

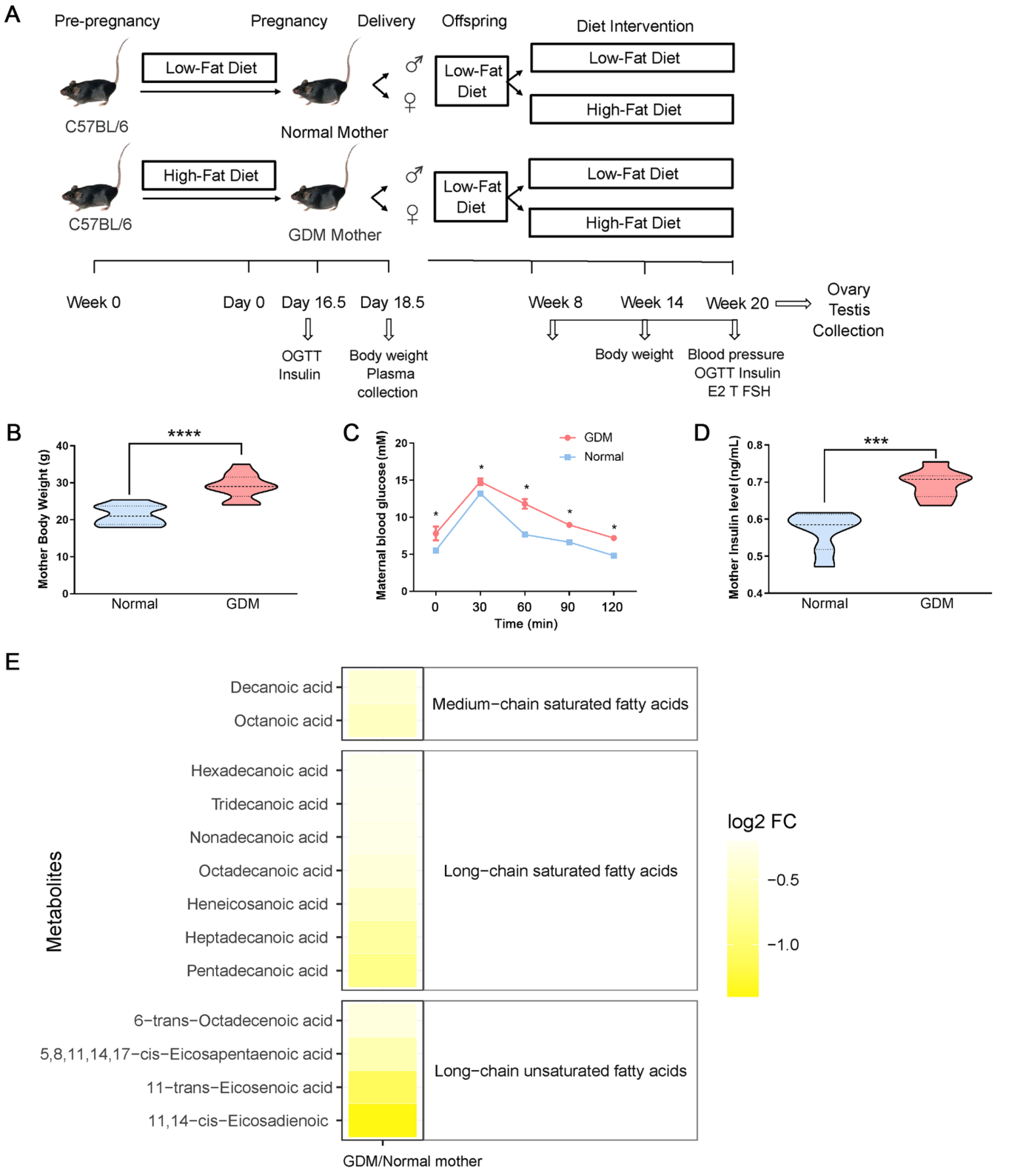

Figure 1. Experimental design and maternal characteristics (A) Graphical display of the experimental design of the study. (B) The body weight of normal mothers ($n=9$) and GDM mothers ($n=13$) in grams at week 14. (C) Oral glucose tolerance test of normal mothers and GDM mothers (blue line and square; $n=13$) at 14 weeks. (D) The plasma insulin levels of normal mothers ($n=9$) and GDM mothers ($n=13$) at week 14. (E) The relative abundances of fatty acids were plotted using $\log_2$ scale. Fold changes of metabolite concentrations compared with their control groups are illustrated in the heatmap. The yellow color indicates decreasing levels. Only the fatty acids with significant P values (Tukey's HSD: $P<0.05$) and q values (FDR: $q<0.05$) are shown. Statistical differences between the normal mother and GDM mother were determined using an unpaired Student's t-test for B and D or a two-way ANOVA followed by a Tukey's post hoc test for C. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) TsingZol (TaKaRa, Dalian, China) was used to isolate RNA and the NanoDrop-2000 spectrophotometer (Thermo Scientific) was utilized to analyze RNA quality. RNA was reversely transcribed to cDNA by using the high-capacity cDNA synthesis kit (TaKaRa) according to the manufacturer's instructions, and PCR was performed using PCR instrument. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [25], and data were normalized to that of *GAPDH* which is the reference gene across all groups of offspring. Primer sequences are listed in Supplementary Table S2.

HE staining

The mice were fasted for 6 h to remove existing intestinal lipid stores. The liver tissue was fixed with 4% paraformaldehyde overnight. After being washed with flow water for 4 h, the tissue was dehydrated with gradually increasing concentrations of ethanol: 70% for 2 h, 80% overnight, 90% for 2 h, and 100% for 1 h, and finally xylene for 30 min. The liver tissue was then embedded with paraffin at 60°C for 2 h, and the paraffin mass was cut into sections of 5 μm. The sections were deparaffinized twice with xylene for 15 min, treated twice with 100% ethanol for 5 min, and then with 95%, 85%, and 75% ethanol (2 min each). The liver sections were stained with hematoxylin (Beyotime) for 5 min, soaked with 1% hydrochloric acid for several seconds, and counterstained with eosin (Beyotime) for 3 min. Finally, the liver sections were dehydrated again with gradually increasing concentrations of ethanol: 75% for 2 min, 85% for 2 min, 95% for 2 min, 100% for 5 min (repeated twice), and xylene for 10 min (repeated twice). Then liver sections were mounted with gum and examined with a Leica DM4000 microscope (Leica, Wetzlar, German) at 20× magnification. Images were collected from liver sections of 3 mice per group for CellProfiler analysis.

Measurement of testis and ovary tissue metabolites by GC-MS

Testes and ovaries were collected from the mice and immediately frozen at −80 °C until the metabolite extraction was performed. Metabolites were extracted from 20 mg of testis or ovary tissues using 2 mL methanol/toluene (1:4 v/v ratio) solution containing 20 μg/mL tridecanoic acid (Nu-Chek Prep, Elysian, USA) and 20 μg/mL nonadecanoic acid (Nu-Chek Prep) as internal standards. Subsequent steps were performed as previously described [26]. The extracted metabolites were analyzed using the Agilent 5977 A MSD system and the Agilent 7890B GC system (Agilent Technologies, Santa Clara, USA). The metabolites were separated using the ReSTEK RTX α-2330 capillary column (100 m, 0.25 mmID, 0.2 μm df, 90% biscyanopropyl/10% phenylcyanopropylpolysiloxane). A total of 1 μL of each sample was injected into the inlet and operated in a split-less mode at 250°C throughout the analysis. The helium pressure was set to a constant flow rate of 1 mL/min.

Metabolite identification and normalization

The GC-MS peaks were deconvoluted using an automated mass spectrometry deconvolution identification system (AMDIS) software. Identification was performed by comparing the peaks' ion fragmentation pattern and retention time (within 20 s window) to an internal lipid mass spectrometry library established using chemical standards [27]. The relative quantification of the identified metabolites was extracted with the most abundant reference ion using MassOmics. Metabolite levels were normalized in the order of internal standards (nonadecanoic acid and tridecanoic acid), batch calibration via quality control (QC) samples, and mass of the reproductive tissues [28].

Statistical analysis

Data are presented as the mean ± SEM. The comparisons between the two groups were conducted using an unpaired Student's *t*-test. Comparisons within four groups (G-L, G-H, N-L, N-H) were determined for both male and female animals using two-way ANOVA and Tukey post-hoc analysis. The tests were performed using GraphPad Software Prism 9 (GraphPad Software, San Diego, USA). The false discovery rates (FDR) were calculated using the q-value function in the R program to account for multiple comparisons. The important variables in the partial least squares discriminant analysis (PLS-DA) projection were determined using the ropls R-package. $P < 0.05$ with corresponding q-value (FDR) < 0.05 were considered statistically significant [29]. A receiver operating characteristic (ROC) curve was constructed using pROC to plot significant metabolites [30]. A graphical representation of the significant metabolites was displayed in heat maps using the gplot and ggplot2 R packages [31]. Correlations between fatty acids (FAs) were determined by nonparametric (Spearman rho) test.

Results

Characteristics and serum fatty acid levels of normal maternal mice and GDM maternal mice

At 18.5 days of gestational age, the normal mice were significantly lighter than the GDM mice (Figure 1B). The blood glucose concentration and insulin level were increased in GDM mice compared to those in normal mice (Figure 1C,D). A total of 13 serum metabolites were significantly different ($P < 0.05$ and q-value < 0.05) between the normal maternal mice and GDM maternal mice (Figure 1E). Lower serum levels of four long-chain unsaturated FAs, 7 long-chain saturated FAs, and 2 medium-chain saturated FAs were found in the GDM maternal mice when compared to those in the normal maternal mice. The principal component analysis (PCA) showed a clear separation in the metabolic profiles between HFD and LFD compositions (Supplementary Figure S1A). A total of 21 metabolites were significantly increased ($P < 0.05$ and q-value < 0.05) in HFD group compared to those in LFD group, including 6 amino acids, 1 short-chain unsaturated FA, 5 medium-chain saturated FAs, 5 long-chain saturated FAs and 4 long-chain unsaturated FAs, while the concentration of three metabolites were reduced, including 1 amino acid, 2 long-chain saturated FAs (Supplementary Figure S1B). Furthermore, the correlation of diet composition (HFD or LFD) with either maternal plasma (GDM mother or normal mother) or offspring testes (fed with HFD or LFD) is depicted in Supplementary Figure S1C. The FAs in HFD group showed significantly positive correlation with GDM maternal plasma, while correlated negatively with testes of offspring fed with LFD. Moreover, the FAs in LFD group exhibited a remarkably positive correlation with offspring fed with LFD, while correlated negatively with testes of offspring fed with HFD.

Characteristics of the offspring

At 20 weeks of age, the body weights of both offspring from the normal mothers or GDM mothers fed with HFD were higher in the

male than in the female within each treatment group. Offspring fed with an LFD from normal mothers had higher body weight in the male than in the female. Male offspring from the normal mothers were significantly heavier if they had followed the HFD after weaning, compared to the LFD group (Figure 2A).

A significantly higher ovary weight was observed in the offspring following an HFD when compared to the LFD group (Figure 2B), while inverse results were observed in testes weights (Figure 2C). Female offspring from normal mothers subsequently fed with an HFD had significantly higher systolic blood pressure when compared to the LFD group (Figure 2D).

In female offspring from normal mothers, a higher blood glucose concentration was found throughout the OGTT experiment in those following an HFD compared to those receiving an LFD. Male offspring from GDM mothers, who were subsequently fed with an HFD had an increased blood glucose level from 0 min to 120 min compared to the LFD group. Whereas, male offspring from normal mothers, who were subsequently fed with an HFD had an increased blood glucose level only at 0 min and 30 min, compared to the LFD group (Figure 2F,G). Plasma insulin levels were elevated in both female and male offspring born to normal mothers and fed with an HFD compared with the LFD group. On the other hand, elevated insulin levels were only observed in male offspring fed with an HFD from GDM mothers when compared to those from normal mothers (Figure 2H,I).

Plasma estrogen concentrations were significantly higher in female offspring from normal mothers, who were subsequently fed with an HFD compared to the corresponding LFD group (Figure 2J). A significant reduction was also observed in plasma testosterone in male offspring from GDM mothers when compared to male offspring from normal mothers (Figure 2L). There was no significant difference in plasma FSH between female and male offspring (Figure 2K,M). Two-way ANOVA statistical results are listed in Supplementary Table S3.

Higher hepatic lipid weight was observed in both offspring fed with HFD from GDM mother than in offspring fed with LFD from GDM mother, with an increase of 25.80 ± 6.18 mg in female offspring and 22.00 ± 8.05 mg in male offspring (Supplementary Figure S2A,B). Two-way ANOVA statistical results are listed in Supplementary Table S4. Similar accumulation of hepatic lipid was observed between female and male offspring, while the offspring

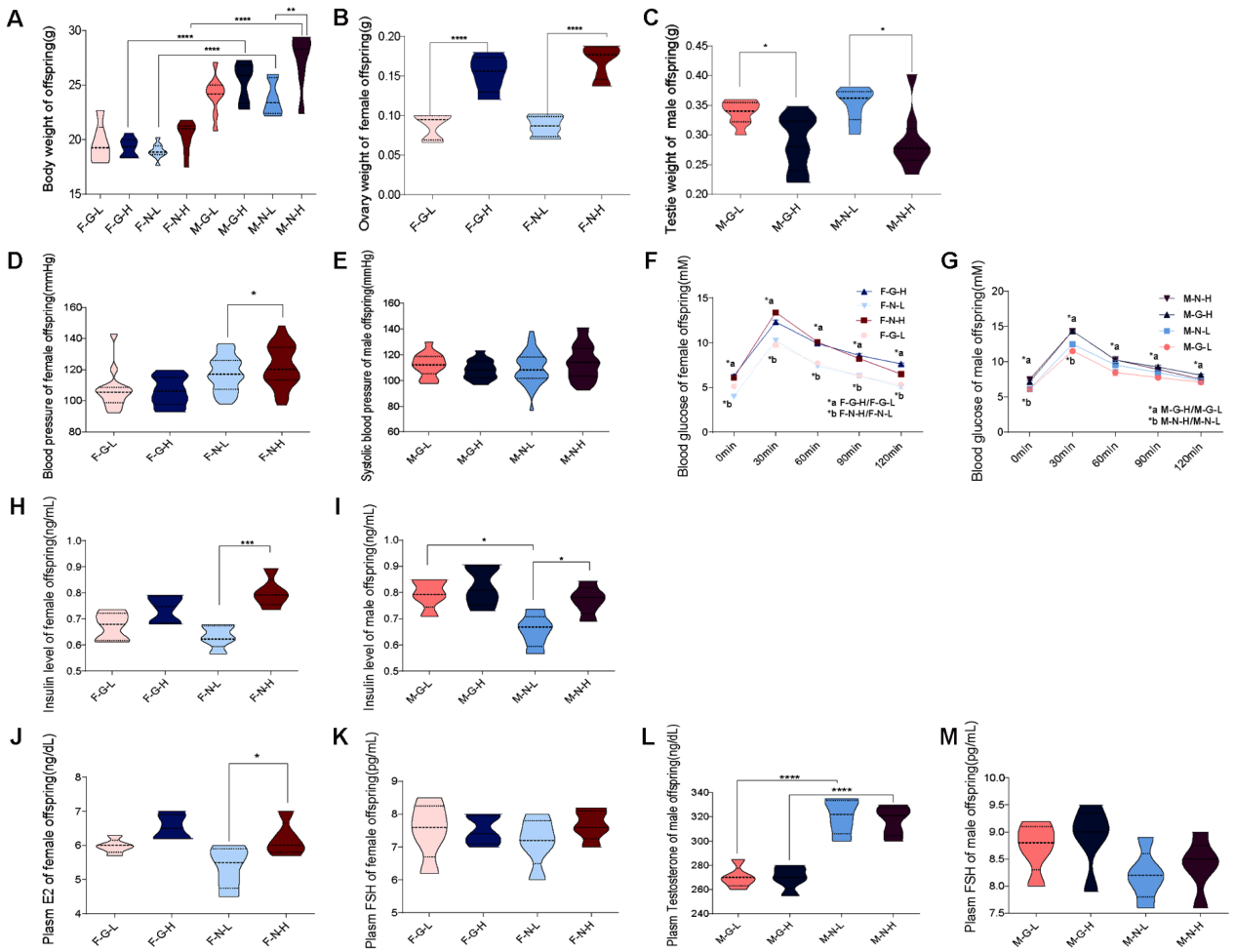

Figure 2. Characteristics of the offspring (A) Body weight of the offspring. (B) Ovary weight of the female offspring. (C) Testis weight of the male offspring. (D) Blood pressure of the female offspring. (E) Systolic blood pressure of the male offspring. (F) OGTT results from the female offspring. (G) OGTT results from the male offspring. (H) Plasma insulin levels of the female offspring. (I) Plasma insulin levels of the male offspring. (J) Plasma E2 levels of the female offspring. (K) Plasma FSH levels of the female offspring. (L) Plasma testosterone levels of the male offspring. (M) Plasma FSH levels of the male offspring. Statistical differences for the characteristics of offspring were determined using a two-way ANOVA followed by a Tukey's post hoc test for A to M. *$P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

bodyweight of the male was higher than that of the female, implying a higher liver weight/body weight ratio in the female offspring than in the male offspring.

Expressions of sex hormone receptors and AKT signaling in the offspring liver and gonadal adipose tissue

The protein expression of estrogen receptor 1 (ESR1) was increased in the ovaries of female offspring fed with an HFD, whereas the protein expression of androgen receptors (AR) was reduced in the testes of male offspring fed with an HFD from normal mothers and in the male offspring fed with LFD from GDM mother (Figure 3A,C). In addition, in male offspring testes, the interactive effect of maternal GDM and offspring HFD showed significance in AR. We measured the mRNA expression of sex hormonal receptors in the offspring reproductive organs. The mRNA expression of ESR1 was significantly increased in the ovary from the female offspring fed with a postnatal HFD from GDM mother, whereas AR expression was significantly reduced in the testis from offspring fed with an HFD (Supplementary Figure S2C,D). Two-way ANOVA statistical results are listed in Supplementary Table S4. Insulin signaling-related molecules are depicted in Figure 3 and Supplementary Table S5. Female offspring exhibited increased AKT signaling activation in the liver in response to an HFD. Meanwhile, maternal GDM increased the insulin signaling in liver (pIRS1, pPI3K) and adipose tissue (pIRS1, pAKT) in the male offspring. The total AKT levels in female liver and male adipose tissue have no significant difference among the 4 groups respectively (Supplementary Figure S2E,F).

Furthermore, we observed a combined effect of maternal GDM and offspring HFD, resulting in elevated expression of TNFα in female and male offspring liver. Notably, in both male and female

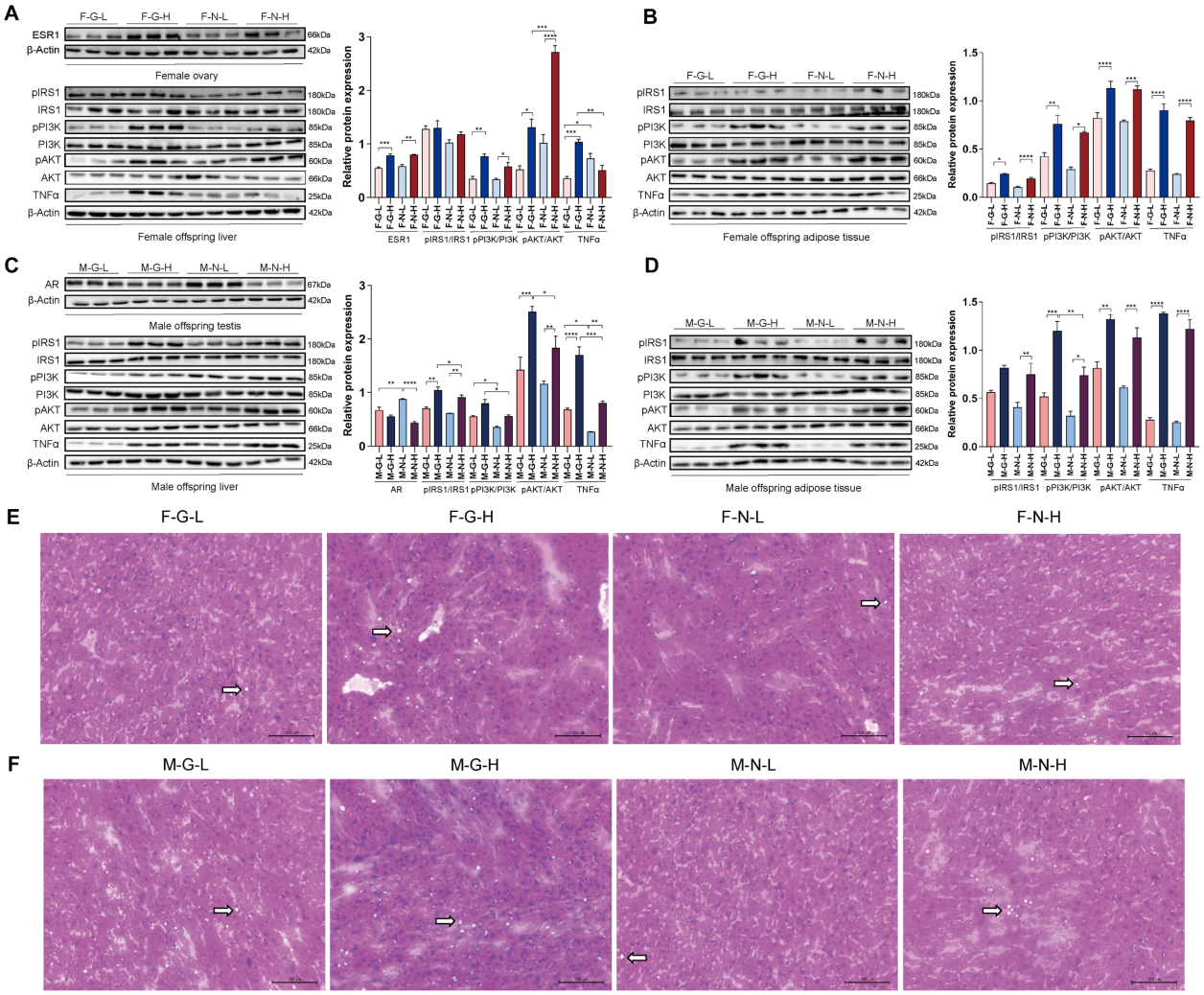

Figure 3. Effects of maternal GDM and offspring HFD on the liver, gonadal adipose tissue, and reproductive organs of the offspring (A) Protein levels of ESR1 in female offspring ovaries (upper panel). Protein levels of pIRS1, pPI3K, pAKT were normalized against total IRS1, PI3K, AKT and TNFα separately in female offspring livers (lower panel). (B) Protein levels of pIRS1, pPI3K, pAKT were normalized against total IRS1, PI3K, AKT separately and TNFα in female offspring gonadal adipose tissue. (C) Protein levels of AR in male offspring testes (upper panel). Protein levels of pIRS1, pPI3K, pAKT were normalized against total IRS1, PI3K, AKT separately and TNFα in male offspring livers (lower panel). (D) Protein levels of pIRS1, pPI3K, pAKT were normalized against total IRS1, PI3K, AKT separately and TNFα in male offspring gonadal adipose tissue. (E) Representative HE-stained liver section images in female offspring. Scale bar = 100 μm. (F) Representative HE-stained liver section images in male offspring. Scale bar = 100 μm. Statistical differences for the characteristics of offspring were determined using a two-way ANOVA followed by a Tukey's post hoc test. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

offspring gonadal adipose tissue, the prominent increase of TNFα was only observed in the HFD groups.

Offspring liver histology

HE-stained sections of liver were evaluated for signs of pathology. Histological evidence showed more lipid droplets in the liver sections of male offspring than in the female offspring in all groups (Figure 3E,F). Only offspring HFD increased the lipid droplets in both female and male offspring liver (Supplementary Figure S2G,H).

Metabolite profiles of offspring ovaries and testes

The principal component analysis (PCA) demonstrated an obvious overlap in the metabolic profile of the offspring ovaries in different groups, while a clear separation was observed in the metabolic profile of the offspring testes. The first three major components of the PCA, i.e., PC1, PC2 and PC3, accounted for 33.9%, 16.2% and 10.5% respectively of the metabolite variation in the ovaries and 33.0%, 11.3% and 7.5% respectively of the variation in the testes (Figure 4A,B). The heatmap showed substantial differences in the lipidomic entities of the offspring ovaries among groups (Figure 4C). Ovaries from offspring born to GDM mothers and fed with an LFD had increased levels of 7 long-chain unsaturated FAs and 1 long-chain saturated FA, and decreased levels of 2 medium-chain unsaturated FAs compared to offspring born to normal mothers and fed with an LFD. A decrease in 4 long-chain unsaturated FAs, 1 long-chain saturated FA, and 1 short-chain saturated FA was observed in offspring born to normal mothers and subsequently fed with HFD, compared to the corresponding LFD group. Interesting, almost no difference was found between maternal GDM in offspring that were fed with HFD and those fed with LFD. The metabolomic analyses of the male offspring testes revealed that the concentrations of all FA and cholesterols were increased in offspring from GDM mothers compared to those in offspring from normal mothers, regardless of their diet after weaning (Figure 4D). Whereas, 2

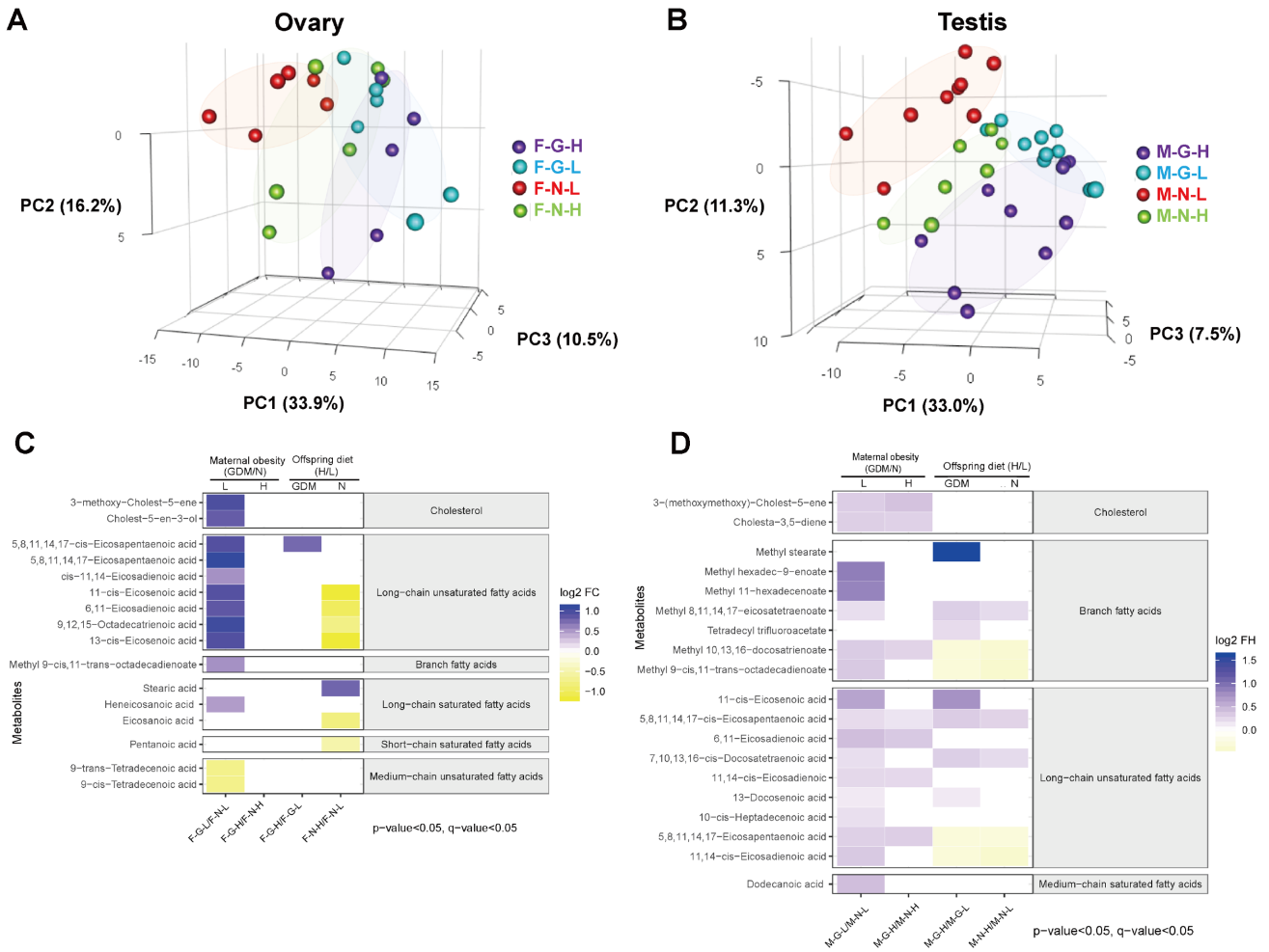

Figure 4. Principal component analysis (PCA) and lipidomic profiles of ovaries and testes in the offspring (A) The PCA analysis of offspring ovaries. (B) The PCA analysis of offspring testes. The color codes of the balls are listed as follows: purple color represents offspring sex (M = male; F = female)-GDM mother-high-fat diet (M/F-G-H); blue color represents offspring sex-GDM mother-low-fat diet (M/F-G-L); red color represents offspring sex-normal mother-high-fat diet (M/F-N-L); green balls represent offspring sex-normal mother-low-fat diet (M/F-N-H). (C) The heatmap demonstrates the female offspring's ovary lipidomic profiles. (D) The heatmap demonstrates the male offspring's testis lipidomic profiles. The maternal obesity indicated that comparisons between the GDM mother normalized against the normal mother (GDM/N) for the offspring fed with the same diet (L = LFD or H = HFD). The offspring diet indicated that comparisons between the offspring HFD normalized against the offspring LFD (H/L) from the same mother (GDM or N). The relative abundances of metabolites were plotted using $\log_2$ scale. Fold changes of metabolite concentrations when compared with their control groups are illustrated by purple color (increasing levels) and yellow color (decreasing levels). Only the metabolites with a significant P value (Tukey's HSD: $P < 0.05$) and q value (FDR: $q < 0.05$) are shown.

branched FAs and 2 long-chain unsaturated FAs were consistently reduced in offspring fed with an HFD compared to those fed with an LFD, regardless of exposure to GDM in utero.

ROC analysis for the metabolic profile of offspring reproductive organs

ROC analysis was performed in the metabolic profiles of offspring ovaries and testes across the groups and an AUC value above 0.95 was considered to be significant. The results showed that 7,10,13,16-*cis*-docosatetraenoic acid in the offspring ovaries exhibited high sensitivity and specificity to discriminate between the diet groups in offspring from normal mothers. The 7,10,13,16-*cis*-docosatetraenoic acid and 11-*cis*-eicosenoic acid in offspring testes could significantly discriminate between the offspring from GDM or normal mothers, subsequently fed with an HFD. The 6,11-eicosadienoic acid, 11-*trans*-eicosenoic acid, 5,8,11,14,17-*cis*-eicosapentaenoic acid, and 13-*cis*-eicosenoic acid in offspring testes could significantly discriminate between the offspring from GDM and normal mothers, subsequently fed with an LFD (Figure 5).

Correlation of metabolic profiles in maternal serum with male offspring testes

Due to the great effect of exposure to GDM in utero and offspring HFD on the male offspring testes metabolome, a correlation analysis was performed with the maternal serum metabolome. In general, offspring fed with an HFD exhibited mostly negative correlations with the maternal serum metabolites in both offspring from GDM and normal mothers. Offspring fed with an LFD showed all positive correlations with the maternal serum metabolites. In particular, a positive correlation was observed between EPA levels in the three different group comparisons (GDM mother vs offspring LFD, normal mother vs offspring LFD, and normal mother vs offspring HFD, Figure 6).

Discussion

It has been demonstrated that maternal GDM and offspring HFD lead to increased blood glucose, insulin signaling and inflammatory response in offspring. In the present study, we found sex differences in either separate or interactive metabolic effects of maternal GDM and offspring HFD on offspring liver, adipose tissue, and reproductive organs. In particular, maternal GDM influences the FA metabolism in male offspring testes, among which both offspring HFD and sexual dimorphism have synergy. These impacts may be associated with the AKT signaling pathway and sex hormones.

We elucidated how PUFAs are altered in offspring reproductive organs with the effects of maternal GDM and offspring HFD. The 7,10,13,16-*cis*-docosatetraenoic acid, an ω-6 PUFA, was elevated in both female offspring ovaries and male offspring testes in response to an HFD. The 7,10,13,16-*cis*-docosatetraenoic acid plays an important role in inflammatory mediation by acting as a ligand for immune receptors and triggering increased TNFα levels [32]. In our study, female offspring fed with a postnatal HFD increased TNFα expression only when they were born to GDM mother. These in-

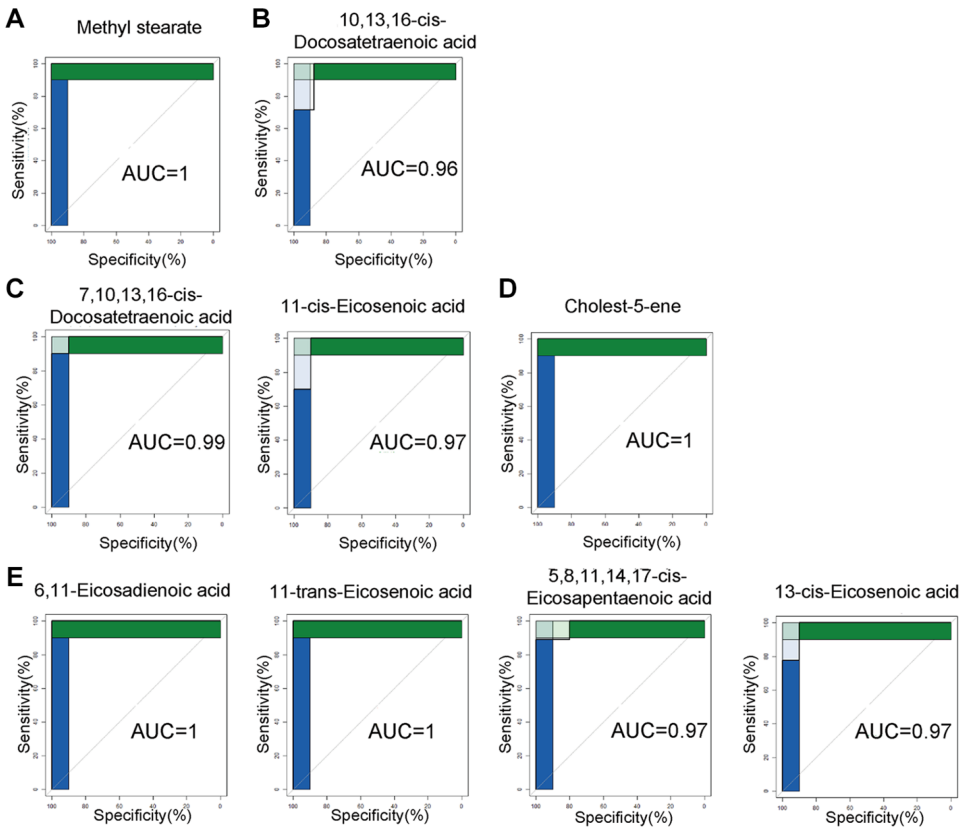

Figure 5. Receiver operating characteristic curves All fatty acids exhibited an area under the ROC curve greater than 0.95. (A) Comparison between F-G-L and F-N-L for methyl stearate. (B) Comparison between F-N-H and F-N-L for 7,10,13,16-*cis*-docosatetraenoic acid. (C) Comparison between M-G-H and M-N-H for 7,10,13,16-*cis*-docosatetraenoic acid and 11-*cis*-eicosenoic acid. (D) Comparison between M-N-H and M-N-L for cholest-5-ene. (E) Comparison between M-G-L and M-N-L for 6,11-eicosadienoic acid, 11-*trans*-eicosenoic acid, 5,8,11,14,17-*cis*-eicosapentaenoic acid and 13-cis-eicosenoic acid. G: GDM mother; N: normal mother; H: high-fat diet; L: low-fat diet.

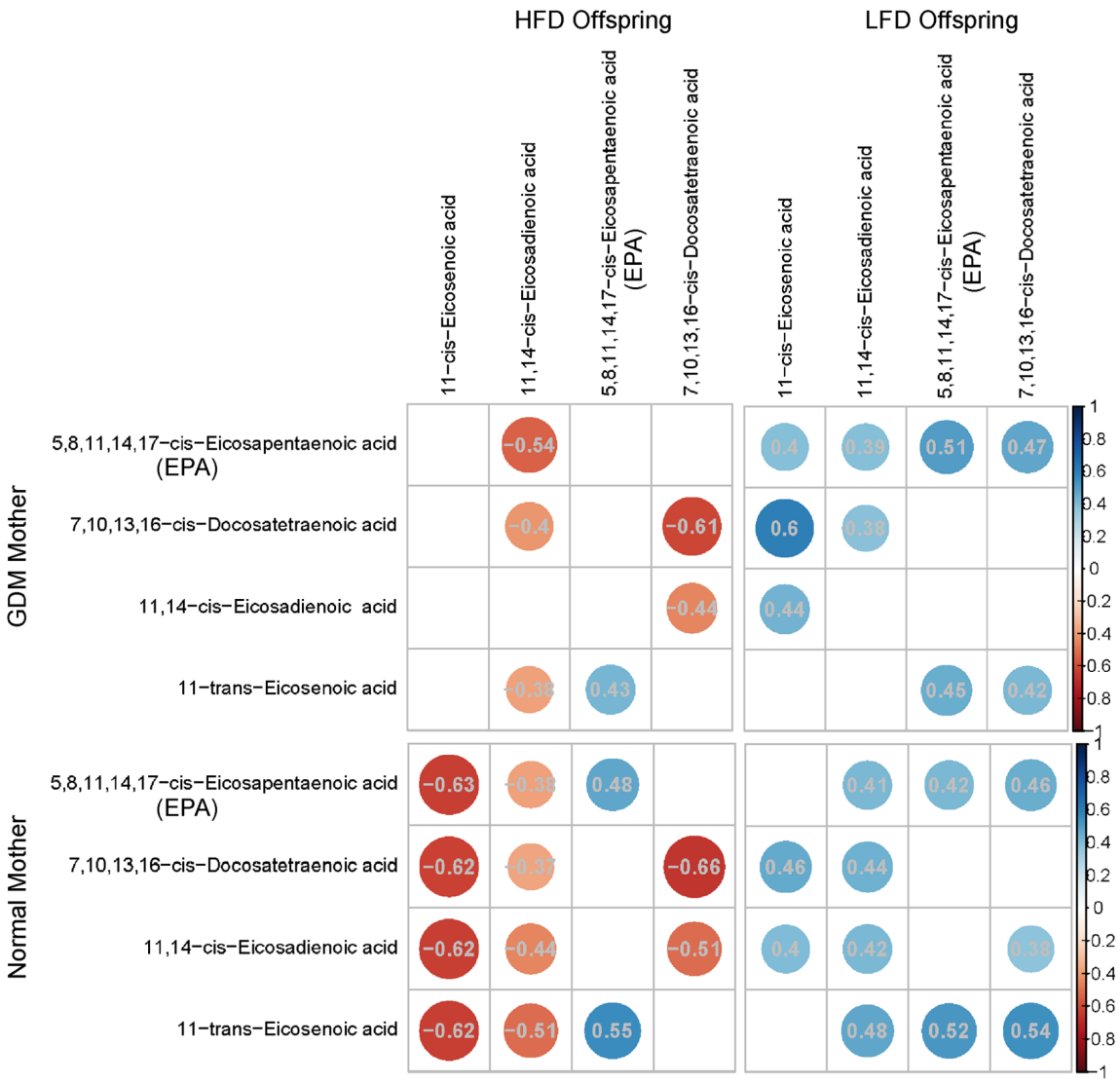

Figure 6. Correlation plots of fatty acids in offspring testes and maternal plasma The blue color represents a positive correlation and the red color represents a negative correlation. The grey numbers are correlation coefficients and only the correlations with *P* values less than 0.05 are colored.

teractions suggest that exposure to GDM in utero may raise the risk of developing inflammation when subsequently exposed to an HFD challenge. Additionally, the maternal GDM strengthened the inflammatory factor TNFα in the male offspring adipose tissue with the synergy of offspring HFD. Similar to our study, prenatal exposure to maternal obesity and HFD programmed the offspring liver toward a pro-inflammatory phenotype, with an upregulation of TNFα [33]. This low-grade inflammation leads to a cascade of events, including inflammatory cell activation, adipocyte growth and dysfunction, and obesity [34,35].

In our GDM mouse model, the reduced concentration of plasma PUFAs was observed. Michael *et al.* [36] demonstrated that the combination of the low milieu of PUFAs and high adiponectin could mediate fetal programming. This evidence suggested that maternal PUFAs may play a potential role in the development of the offspring. Insulin has been shown to modulate the expressions of FA synthase and stearoyl-CoA desaturase, and the latter catalyses FA synthesis [37]. The fetal liver and adipose tissue are often directly affected by altered *in-utero* conditions [38]. Liver and adipose tissue play a significant role in the whole-body insulin action [39]. In our mouse model, insulin signaling increased by maternal GDM was detected in male offspring liver and adipose tissue. Studies on the effects of maternal obesity on both male and female offspring have frequently elucidated that male offspring have a more pronounced detrimental phenotype, including adiposity and impaired islet function [40,41]. Indeed, the insulin signaling was elevated in the female offspring fed with an HFD. The impact of maternal GDM is inconspicuous in our study. Yokomizo and colleagues [42] showed that plasma estradiol levels from HFD-induced obesity mothers were higher in female offspring than in male offspring, providing evidence that females may be protected from deficient insulin level in the maternal HFD state. It should be noted that female but not male offspring appear to be primed to cope with a nutritionally rich in utero environment, which may lead to differences in future obesity risk.

The decreased concentration of eicosapentaenoic acid (EPA) was observed in the serum of maternal mice with GDM. EPA is an essential fatty acid [43] that must be delivered by placental transfer to serve as an important substrate for fetal development [44]. Ac-

cordingly, an abnormal level of maternal circulating fatty acids may result in adverse maternal-fetal interactions and affect offspring phenotype. Interestingly, the concentration of EPA was also attenuated in male offspring testes from GDM mothers and fed with a postnatal HFD. Notably, we found that in male offspring liver exposure to maternal GDM contributes to the increased insulin signaling only when given an HFD again, implying that GDM exacerbates the impacts of a postnatal HFD challenge. This result is consistent with another recent study demonstrating that post-weaning fat exposure promotes glucose intolerance and compromises insulin-stimulated glucose uptake, which is aggravated by maternal fat exposure [19]. On the other hand, the correlation plots of FA between offspring testes and maternal plasma suggest that the correlation is likely with the offspring diet. We also observed that an LFD was associated with better outcomes for the offspring and was positively correlated with PUFAs. Indeed, PUFAs have been reported to reduce chronic inflammation and have potential anti-obesogenic effects [45,46]. Taken together, we can conclude that the reduced PUFA levels are involved in the combined effect of maternal GDM and offspring HFD on offspring reproductive organs, leading to offspring obesity later in life.

The obesogenic consequences resulted from the insulin signaling activation are partially associated with the sex hormone levels in the male offspring. Fetal programming by maternal GDM has been proposed as a predictive-adaptive response to in utero conditions [47]. Our study seems to be the case with male offspring, particularly influencing the offspring sex hormone. The male offspring in this study who were born to GDM mothers had reduced testosterone levels and decreased androgen receptor (AR) expression in their testes. There is considerable evidence that testosterone deficiency is involved in the pathological changes in body fat composition by causing the onset of visceral obesity and subsequently contributing to insulin signaling activation [48]. In addition, previous research demonstrated that androgen may exhibit sex-specific effects on the body fat composition through the FABP4-PPAPγ pathway [49] and further mediate the PUFAs content [50]. Interestingly, a higher liver weight/body weight ratio was observed in the female offspring with HFD. Previous studies showed that female mice are more prone to hepatic lipid storage than male mice. Schiffrin *et al.* [51] suggested that it might be related to the female-specific overexpression of genes (e.g. *G0s2*, *Plin2*, *Scd1*) involved in lipid storage. Della *et al.* [52] also reported that females could effectively utilize dietary and available amino acids in the liver by ERα-dependent signaling pathways. The possible reason for the female mice to exhibit a higher insulin level than the male mice is that the female mice express higher levels of estrogen receptors to promote insulin sensitivity in response to HFD intervention [53]. Moreover, previous studies also demonstrated that estrogen improved the ability of insulin to regulate hepatic and peripheral glucose metabolism in HFD-induced obesity in female mice with ovariectomy [54]. Overall, the maternal GDM and offspring HFD could cause diverse metabolic consequences in the female and male offspring which might be related to sex hormones.

In summary, we confirmed that GDM aggravates the effects of a postnatal HFD in male offspring, and increases the risk of developing inflammation in female offspring exposed to an HFD. Importantly, maternal GDM affects FA metabolism with the synergistic effect of offspring HFD in male offspring testes related to the impacts of sex hormones and the increase in insulin signaling. PUFAs might have the potential to be the new therapeutic target for preventing the detrimental effects of GDM on male offspring.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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