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Sexual Dimorphism in the Fetal Cardiac Response to Maternal Nutrient Restriction

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Abstract

Poor maternal nutrition causes intrauterine growth restriction (IUGR); however, its effects on fetal cardiac development are unclear. We have developed a baboon model of moderate maternal undernutrition, leading to IUGR. We hypothesized that IUGR affects fetal cardiac structure and metabolism. Six control pregnant baboons ate *ad-libitum* (CTRL) or 70% CTRL from 0.16 of gestation (G). Fetuses were euthanized at C-section at 0.9G under general anesthesia. Male but not female IUGR fetuses showed left ventricular fibrosis inversely correlated with birth weight. Expression of extracellular matrix protein TSP-1 was increased ($p < 0.05$) in male IUGR s. Expression of cardiac fibrotic markers TGF β , SMAD3 and ALK-1 were downregulated in male IUGRs with no difference in females. Autophagy was present in male IUGR evidenced by upregulation of ATG7 expression and lipidation LC3B. Global miRNA expression profiling revealed 56 annotated and novel cardiac miRNAs exclusively dysregulated in female IUGR, and 38 cardiac miRNAs were exclusively dysregulated in males ($p < 0.05$). Fifteen (CTRL) and 23 (IUGR) miRNAs, were differentially expressed between males and females ($p < 0.05$) suggesting sexual dimorphism, which can be at least partially explained by differential expression of upstream transcription factors (e.g. HNF4 α , and NF κ B p50). Lipidomics analysis of fetal cardiac tissue exhibited a net increase in diacylglycerol and plasmalogens and a decrease in triglycerides and

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phosphatidylcholines. In summary, IUGR resulting from decreased maternal nutrition is associated with sex-dependent dysregulations in cardiac structure, miRNA expression, and lipid metabolism. If these changes persist postnatally, they may program offspring for higher later life cardiac risk.

Keywords

maternal undernutrition; miRNA; lipidomics; cardiac fibrosis; sexual dimorphism; autophagy

1. Introduction

The “fetal origins of disease” hypothesis proposes that maternal undernutrition causes permanent structural and metabolic changes in developing organs that increase the subsequent risk of cardiovascular and metabolic disease [1]. Poor maternal nutrition during pregnancy is thought to account for 14% of intrauterine growth restriction (IUGR) in developing countries (4th Report on The World Nutrition Situation - ACC/SCN 2000) while other important etiologic factors such as malaria, acute and chronic infections and cigarette smoking could contribute as well. In developed countries, smoking is the most important determinant of IUGR, followed by factors such as maternal nutrition, pre-eclampsia, genetic factors and alcohol or drug use[2].

The cellular processes by which prenatal maternal nutritional challenges predispose to later life offspring disease are termed fetal or developmental programming. Programming can be defined as the responses of the developing organism to a challenge during a critical window of development that alter offspring phenotype with potential persistent effects on life course health. The first human epidemiological studies on programming reported life course cardiovascular disease associated with poor fetal growth in both males and females [3, 4]. Similarly, numerous animal studies addressed the short- and long-term consequences of poor maternal nutrition for the offspring health [5]. Rat and mouse models are clearly the preferred nonhuman models and both have shown that long-term effects of adverse maternal nutrition include changes in postnatal growth [6] and altered metabolism in offspring [7-11]. Maternal low protein diet results in high blood pressure in offspring [10], altered endocrine function [9], immune function [7], and renal development [11]. Same long-term consequences of maternal malnutrition were observed in long-gestation, precocial species including r, sheep [12, 13] and nonhuman primates [14-16]. The pig is currently considered a reliable large animal model for translational research in IUGR as result of deficiencies in maternal nutritional supply or placental development [17]. In pigs, IUGR offspring have compromised health, reduced growth potential and high predisposition for adiposity [18].

Few published studies focused on effect of maternal nutrition on myocardial development clearly show that the morphology and the metabolism of the heart are sensitive to the intrauterine insults. For example, Dong et al. have reported that maternal nutrient restriction in sheep results in enlarged ventricular size associated with an upregulation of insulin-like growth factor (IGF) receptor [19]. Using the same model of maternal nutrient restriction, Han et al. have reported significant changes in cardiac gene expression measured by hybridization assay [12]. Gilbert et al have demonstrated a dysregulation of the renin-

angiotensin system in midgestation left ventricle of fetal sheep exposed to 50% reduction in maternal nutrition [13]. Beauchamp et al. [20] showed impaired cardiac muscle energetics as result of maternal undernutrition and low birth weight. Maternal nutrient restriction in rats results in decreased cardiomyocytes number [21], whereas the rat model of maternal protein restriction showed an increase in myocardial expression and deposition of collagens and alters the expression of pro-fibrotic genes in adult offspring [22].

We have developed a baboon model of moderate maternal nutrient reduction during pregnancy that produces IUGR offspring [14, 23-30]. In this model, throughout pregnancy, undernourished mothers ate 70% of the diet consumed by controls – a 30% reduction in intake. We have previously shown that the offspring are growth restricted at birth, with male and female offspring of restricted mothers weighing approximately 11% less than offspring of controls [25]. Metabolic, functional, and epigenetic abnormalities were reported in the fetal and/or neonatal brain [15], kidney [28] and liver [14, 23] of IUGR baboons. In adult life, the IUGR baboons showed an increase in insulin resistance [31] and engage in more aggressive and affiliative behaviors compared to control animals [32]. In this study, we sought to determine how maternal undernutrition affects fetal cardiac development.

2. Materials and Methods

2.1 Animal care and maintenance

All procedures were approved by the Texas Biomedical Research Institute (Texas Biomed) Institutional Animal Care and Use Committee and conducted in AAALAC-approved facilities. Thirty-eight female baboons (*Papio hamadryas anubis*) from the Southwest National Primate Research Center at San Antonio, Texas, USA were recruited for this study and maintained in group housing. The caging system allows control and monitoring of food intake while still permitting normal social and physical activity and has been described in detail [14, 29, 30]. Briefly, groups of 16 females carefully selected to produce a homogeneous group were assembled and socialized in the presence of a vasectomized male while eating Purina Monkey Diet 5038 (Purina, St. Louis, Missouri, USA) *ad libitum*. After acclimation, the vasectomized male was replaced by a proven breeder male. Females were observed for turgescence (sex skin swelling) and signs of vaginal bleeding to enable timing of pregnancy [33]. Pregnancy was confirmed by ultrasound at 0.16G after which they were randomly assigned to either a control group of females that continued to receive ad lib feed (n=24) or a group that underwent maternal nutrient restriction and received 70% of the feed eaten by controls on a weight- adjusted basis (n=14). The system of individual feeding of adult baboons maintained in an outdoor group social environment has been previously described in detail [14].

2.2 Study Design

Briefly, once a day prior to feeding, all baboons were placed in individual feeding cages. Baboons passed along a chute, over a scale, and into an individual feeding cage. The weight of each baboon was obtained as it crossed an electronic scale system (GSE 665; GSE Scale Systems, Livonia, MI, USA). The weight recorded was the mean of 50 individual measurements over 3s. If the SD of the weight measurement was >1% of the mean weight,

the weight was automatically discarded, and the weighing procedure was repeated. Once housed in an individual cage, each animal was fed between either 07:00 and 09:00 or 11:00 and 13:00. Water was available continuously in the individual feeding cage and the group cages. Animals were fed Purina Monkey Diet 5038 (Purina, St. Louis, MO, USA), described by the vendor as “a complete life-cycle diet for all Old World Primates.” The biscuit contains stabilized vitamin C as well as all other required vitamins. Its basic composition is crude protein 15 %, crude fat 5%, crude fiber 6%, ash 5%, and added minerals 3%. At the start of the feeding period, each baboon was given 60 biscuits in the feeding tray of the individual cage. At the end of the 2 h feeding period, the baboons were returned to the group cage. Biscuits remaining in the tray, on the floor of the cage, and in the pan beneath the cage were counted. Food consumption of animals, weights, and health status were recorded each day.

2.3 Cesarean sections

Surgical procedures were performed by a fully certified MD or DVM, and postsurgical care was prescribed and monitored by a primate center veterinarian. Cesarean sections were performed at 165d G (0.9G) using standard techniques that have been previously described in detail [22]. All baboons were premedicated with ketamine hydrochloride (10 mg/kg, i.m.). After intubation, isoflurane (2%) was used to maintain a surgical plane of anesthesia throughout surgery. Following hysterotomy, the umbilical cord was identified and used for fetal exsanguination while under general anesthesia as approved by the AVMA Panel on Euthanasia. The placenta and fetus were removed from the uterus and immediately submitted for morphometric analyses and tissue sampling. Fetal morphometrics was obtained at necropsy. Post-operative maternal analgesia was by buprenorphine hydrochloride (Buprenorphine HCl Injection, Hospira, Inc., Lake Forest, IL) 0.015 mg/kg/d split as two doses for 3 days. After recovery in individual cages, mothers were returned to their group housing.

2.4 Materials

Antibody against HNF4 α was purchased from Origene. Antibody against ATG7 and LC3B were part of the Autophagy Antibody Sampler Kit from Cell Signaling Technologies (Danvers, MA, USA). TGF β , SMAD3, and NF κ B p50 were also purchased from Cell Signaling. The anti-TSP1-1 antibody was from R&D Systems and anti-CTGF – from GeneTex Inc. The anti-ALK1 and anti-PPAR γ antibodies were purchased from Santa Cruz Biotechnology, and antibody against β -Actin - from Sigma (St. Louis, MO, USA).

2.5 Histology

The excised hearts were washed in PBS, fixed overnight in 4% paraformaldehyde, and embedded in paraffin. After serial sectioning of hearts, (apex to base) 7- μ m sections were stained either with hematoxylin-eosin to visualize tissue architecture or with Masson trichrome to visualize the fibrotic tissue. Areas of fibrosis within sections were measured by visualizing blue-stained areas, exclusive of staining that colocalized with perivascular or intramural vascular structures. Using ImageJ software (<http://rsbweb.nih.gov/ij/>), blue-stained areas, and non-stained myocyte areas from each section were determined using

color-based thresholding. The percentage of total fibrosis area was calculated as the summed blue-stained areas divided by total ventricular area, as described previously [34].

2.6 RNA isolation

Overall, 12 fetal hearts were studied: 6 from baboons born to mothers fed *ad libitum* (n=3 males and 3 females) and 6 from baboons born to undernourished mothers (n=3 females and 3 males). Total RNA was isolated with the use of RNAeasy kit (Qiagen). The integrity of RNA was tested by spectroscopic analysis and by resolving on denaturing formaldehyde gel.

2.7 miRNA profiling

RNA quality control, profiling and data analysis were performed by LC Sciences, LLC (Houston, TX) as previously described [35]. RNA samples were initially sequenced [35] and then custom-made microarrays were used to profile the expression of fetal cardiac miRNAs in all 12 RNA samples (6 CTRL and 6 IUGR). The GEO accession number for the microarray data is **GSE69609**.

2.8 Western blot analysis

The whole cell homogenates were isolated and resolved on 4-20% gradient SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes; the membranes were blocked with 5% skim milk in Tween-20/Tris-buffered saline and probed with antibodies. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies. Finally, the enhanced chemiluminescence (ECL) reaction was performed and the blots were visualized by G-Box (Syngene). The intensity of each signal spot was transformed into digital data with auto-background subtraction during spot density analysis using the Syngene GeneTools software.

2.9 Lipidomics Analysis

Samples were extracted as described in detail previously [36, 37]. Briefly, fetal cardiac tissues were homogenized in cold methanol using a tissue homogenizer (Bullet Blender), and then water and chloroform were added to get a final proportion of chloroform/methanol/water of 2/1/0.6 (v/v/v). The sample was then vigorously vortexed, incubated on ice for 5 min and centrifuged for 10 min at 10,000 rpm and 4 °C. The bottom organic phase was collected into autosampler vials, dried in a vacuum centrifuge, and dissolved in methanol for the liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis. The LC-MS/MS data were collected in both negative and positive modes as detailed elsewhere [38] using an UPLC system (NanoAcquity, Waters) connected to an Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Fisher Scientific) employing both HCD (high-energy collision dissociation) and CID (collision-induced dissociation) fragmentation. The LC-MS/MS data were analyzed with LIQUID [39] and each of the identified lipid species were manually validated. Identified lipids were quantified using MZmine 2 to extract peak intensities. [40] The data were then log2 transformed, to reduce heteroscedasticity, and normalized by linear regression and central tendency using InfernoRDN, formerly DAnTE. [41]

2.10 Statistical analysis

Data are reported as mean $\pm$ SEM. The effects of maternal undernutrition and fetal sex were evaluated using a two-way ANOVA with Tukey's Honest Significant Difference (HSD) as the post hoc procedure. $P < 0.05$ was considered as significant. Lipidomics data were analyzed using Welch's t-test.

3. Results

3.1 Fetal morphometrics

At the time of recruitment to the study non-pregnant female baboons were randomly assigned to the control group weighed 16.7 ± 0.43 kg ($n=24$) and those assigned to the nutrient restriction group weighed 16.3 ± 0.77 kg ($n=14$; $p > 0.05$). Within the complete groups of animals studied, the maternal nutrient reduction caused intrauterine growth restriction (IUGR): the average birth weight in IUGR fetuses ($n=14$) was 715 ± 21.0 g ($p < 0.03$) vs. 795 ± 23.5 g in CTRL ($n=24$). When divided by sex, male control fetuses were heavier than female control fetuses (840 ± 33.6 g, $n=11$ and 757 ± 30.0 g, $n=13$ respectively, $p < 0.05$). Male IUGR fetuses (749 ± 26.5 g, $n=8$) were lighter than male CTRL fetuses ($p < 0.05$), and female IUGR fetuses (671 ± 26.0 g, $n=6$) were lighter than female CTRL fetuses ($p < 0.05$). Thus, data show that moderate maternal nutrient reduction produced a similar degree of IUGR in males (9.8%) and females (12%). Morphometric measures of animals in the subset in which fetal hearts were analyzed are presented in Table 1. There was a significant reduction in maternal body weight as result of undernutrition in the female carrying mothers compared to controls. There was a tendency to decrease in body weight in the undernourished dams with male fetuses, which, however, did not reach a statistical significance ($p=0.9$). When measured in absolute values, both male and female IUGR fetuses had a decrease in placental weight compared to control fetuses. In addition, female but not male IUGRs showed a significant reduction in kidney, liver and lung weights compared to male CTRLs. When analyzed by sex, female CTRL fetuses had a lighter brain and heavier lungs compared to male CTRLs, whereas female IUGRs had lighter placenta, heart, and kidney compared to their male counterparts. When normalized to body weight, only lungs were lighter in male IUGRs compared to male CTRLs. No differences in heart weight to body weight ratio were observed. When divided by fetal sex, the lungs and the brain were significantly heavier in female CTRLs and IUGRs respectively compared to males within the same group.

3.2 Fibrosis and TGF β signaling in IUGR hearts

No structural abnormalities were observed by H&E staining (Figure 1A). However, Masson trichrome staining of cardiac sections (Figure 1A) showed an increased myocardial fibrosis in male IUGR fetuses. There was an inverse correlation between the degree of cardiac fibrosis and birth weight (Figure 1B, $r=-0.83$, $p=0.02$, $n=6$). No accumulation of fibrotic tissue was detected in female control or IUGR fetuses. Tissue fibrosis is regulated by a family of structurally unrelated macromolecules called matricellular proteins, which are generally not expressed in the healthy heart but are markedly increased in the remodeling myocardium [42]. Thrombospondin (TSP)-1 is a prototypical matricellular protein that is not part of the normal cardiac matrix network but is upregulated during myocardial infarction

[43] or pressure overload [44]. TSP-1 is a pro-fibrotic factor and a key activator of transforming growth factor (TGF)- β [44]. Western blot analyses show a significant increase in cardiac TSP-1 level in male but not female IUGR ($p=0.001$, Figure 2A-B). There was a tendency to increase in TSP1 levels in female CTRL vs male CTRL, which did not reach a statistical significance ($p=0.16$). Surprisingly, the expression of TGF β was decreased only in male fetuses (4.7-fold, $p=0.03$, Figure 2D).

Smad3/TGF β signaling has been previously shown to mediate cardiac fibrosis in cardiovascular remodeling associated with myocardial infarction [45] and diabetes [46]. Consistent with the observed TGF β levels, we found a significant reduction in cardiac SMAD3 level ($p=0.03$, Figure 2C) in male IUGR fetuses compared to controls female control and in IUGR were similar. The activin receptor-like kinase-1 (ALK1) is a type I cell-surface receptor for the TGF- β family of proteins and has been shown to play an important role in cardiovascular remodeling [47, 48]. The protein levels of cardiac ALK1 were decreased in male IUGR baboons ($p=0.03$), while female control and IUGR were similar. Again, there was a tendency to increase in the protein levels of SMAD3 and TGF β in the female IUGRs vs. male IUGR, which did not reach a statistical significance. Both TGF β and ALK1 showed statistically significant interaction effect suggesting that the effect of maternal undernutrition on the expression of these proteins was fetal sex-dependent ($p=0.01$ and 0.001 respectively). Since Connective Tissue Growth Factor (CTGF) is an important downstream effector of the pro-fibrotic actions of TGF β [49], we examined whether IUGR also results in activation of CTGF signaling. No differences, however, in CTGF level were observed across the groups (Figure 2E).

3.3 Autophagy is activated in the IUGR hearts

The heart responds to nutrient stress by activation of autophagic pathways [50]. We, therefore, measured the levels of autophagy-related protein (ATG7) and a ratio of microtubule-associated protein 1B-light chain 3 (LC3B)-II to LC3B-I, two reliable markers of autophagy [51] (Figure 3). There was a significant increase in the protein level of cardiac ATG7 in the male but not female IUGRs ($p=0.02$, Figure 3A-B). In CTRL animals, the cardiac expression of ATG7 was significantly greater in females compared to males ($p=0.04$). The ratio of LC3B-II to LC3B-I, a marker representing an autophagic flux at a given point of time [52], showed a tendency to increase in male IUGR, however, did not reach a statistical significance ($p=0.09$, Figure 3C).

3.4 Expression of cardiac miRNAs is affected by maternal undernutrition

We have previously sequenced baboon cardiac miRNAs and identified fetal miRNAs affected by maternal obesity [35]. In this study, we sought to determine whether fetal cardiac miRNAs are affected by maternal undernutrition. We found that overall 63 miRNAs were differentially expressed in female IUGR fetuses when compared to female controls (Figure 4, $p<0.05$). Of them, 17 were upregulated and 46 downregulated. About 19 differentially expressed miRNAs were mapped to human miRNAs, and eight of them (*hsa-miR-1*, *-30c*, *-127*, *-143/145*, *-181b*, *-378*, *-455* and *let-7a*) were previously linked to cardiovascular diseases (CVD) [53-60]. In male fetuses, 56 miRNAs were differentially expressed with IUGR ($p<0.05$, Figure 4), 12 of them were upregulated and 44 downregulated. Overall, 26

miRNAs were mapped to human; fourteen of them (*hsa-miR-1*, *-26a*, *-27b*, *-28*, *-30e*, *-98*, *-99b*, *-143*, *-148a*, *-181a*, *-208b*, *-299-3p*, *-378*, and *-483*) were previously linked to CVD [53, 61-72]. Only three of differentially expressed miRNAs - *hsa-miR-1*, *-378*, and *-143* - were common for male and female IUGR fetuses and showed a significant reduction with IUGR vs. control. No correlation between miRNA expression and the birth weights were observed (not shown).

We also detected several 22-nucleotide-long sequences that were not annotated to any known miRNAs but appeared significantly dysregulated by maternal undernutrition in both male and female fetuses. Potential candidate (PC) miRNAs with the highest amplitude of change in males and females are presented in Supplemental Tables 1 and 2 respectively.

3.5 Sexual Dimorphism in miRNA Expression

There were differences in expression of fetal cardiac miRNAs between male and fetal fetuses within both control and IUGR groups (Figure 5). In control fetuses, twelve miRNAs were increased and three decreased in males compared to females ($p < 0.05$). The miRNAs with the highest amplitude of change included *miR-1*, *-455*, *-145*, *-30c* and *let-7* (from 4 to 5.5-fold increase, $p < 0.05$, Figure 5A). In contrast, 23 cardiac miRNAs were differentially expressed in male IUGRs compared to female IUGR fetuses (Figure 5B). Using the CHIPBase software (Sun Yat-sen University, Guangdong, China), we scanned the 1.5 kb of genomic sequence located upstream of the predicted start sites of miRNAs differentially expressed between male and female fetuses in each group.

In control fetuses, we identified common putative consensus binding sites for hepatocyte nuclear factor 4 alpha (HNF4 α). In IUGR baboons, the miRNAs whose expression has been affected by fetal sex shared common binding sites for nuclear factor NF κ B p50 and Caudal Type Homeo Box Transcription Factor 2 (CDX2). Western Blot was performed to compare the expression of HNF4 α , NF κ B p50 and CDX2 levels in control and IUGR male and female hearts (Figure 5C-F). Consistent with miRNA expression data, the level of HNF4 α was significantly greater in male CTRL baboon fetuses compared to females ($p = 0.04$), while no fetal sex-dependent differences were observed in IUGR baboons. The expression of NF κ B p50 was significantly downregulated in female CTRLs compared to male CTRLs ($p = 0.02$), and significantly upregulated in female IUGRs compared to male IUGRs ($p = 0.02$). There was also a statistically significant interaction effect suggesting that the effect of maternal nutrition on expression of NF κ B1 was sex-dependent ($p = 0.01$). No differences in CDX2 were observed across the groups.

3.6 Pathways Analysis

Several databases were utilized to search for predicted targets of differentially expressed miRNAs: Target Scan 5.0, miRDB, and DIANA LAB. Using more than 300 targets labeled as “experimentally observed” and that are expressed in heart, we have conducted a pathway analysis using the Ingenuity Pathway Analysis (IPA), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to reveal signaling pathways potentially affected by IUGR (Supplemental Table 3).

Consistently with our data, the TGF β signaling was among the highly affected pathways ($p=6.48E-10$). The analysis identified PI3K-Akt signaling as most affected in males and females in response to maternal undernutrition ($p=6.55E-13$ and $2.40E-10$ respectively). Other affected pathways included p53 signaling and Ubiquitin-mediated proteolysis. Several pathways were changed in a fetal sex-dependent manner: for example, regulation of actin cytoskeleton, and apoptosis signaling were in female but not male fetuses. Using “*Comparison Analysis*” tool from IPA, we next compared the cardiotoxicity-associated pathways between male and female IUGRs (Supplemental Figure). Consistent with our observations, *cardiac fibrosis*-related pathways were significantly increased in male vs. female IUGR. Furthermore, several cardiac disease-associated categories such as *cardiac inflammation*, *cardiac infarction*, and *cardiac arteriopathy* were solely activated in males while no changes were observed in females.

3.7 Cardiac lipid profiles of control and IUGR baboons

Previous studies indicate that intrauterine malnutrition is associated with affected lipid metabolism in the offspring [73, 74]. Furthermore, associations between small birthweight and a less favorable lipid profile have been described [75, 76]. We applied a lipidomics approach to profile and contrast the fetal cardiac lipidome of male and female control and IUGR baboons.

About 300 lipids were identified in the fetal hearts of six IUGR and six control animals. Figure 6 and Supplemental Table 4 show a detailed list of metabolites detected with statistically significant changes ($p<0.05$). We observed a dramatic remodeling in the pool of diglycerides (DG), triglycerides (TG), plasmalogens, phosphatidylcholines (PC), and phosphoethanolamines (PE). Both long chain- and very long-chain DGs were significantly upregulated with an average increase of +180%. All but one (PC 16:0-18:1) shorter chain PCs were significantly reduced in IUGR hearts compared to controls, with an average decrease of -60%. Long chain PCs were also dysregulated but with no consistent pattern. Specifically, ten long chain PCs (PC(18:0/18:1); PC(18:1/18:1); PC(18:0/18:2); PC(18:2/0:0); PC(18:3/22:6); PC(20:4/20:5); PC(20:3/0:0); PC(20:3/20:4); PC(18:1/22:6); PC(22:4/22:6)) were significantly increased in the IUGR hearts vs control, while eight other PC were decreased (PC(18:2/0:0); PC(18:3/22:6); PC(20:4/20:5); PC(20:3/0:0); PC(20:3/20:4); PC(18:1/22:6); PC(22:4/22:6); PC(34:4)) leading to a net increase of 109%. Striking changes were observed in the pool of plasmalogens: eight of them (PE(P-16:0/20:1) (co-eluted with PE(P-18:0/18:1)); PE(P-16:0/20:3); (co-eluted with PE(P-18:0/18:3)); as well as PE(P-16:0/20:4); PE(P-18:0/20:3); PE(P-18:0/20:4); and PE(P-18:1/22:6)) showed a significant upregulation in the IUGR hearts compared to controls with an average change of 182%.

The intensities of five triacylglycerols TG(54:6); TG(56:9); and TG(17:1/18:1/18:2), including two coeluting TG(16:1/20:4/22:6) and TG(18:2/18:3/22:6) were significantly decreased in IUGR hearts compared to controls, while only TG(50:1) was increased. Three different dysregulated sphingomyelins (SM) were detected: one of them SM (d18:2-14:0) was decreased in IUGRs ($p=0.0008$), and other two co-eluting SM(d18:1-16:0) and SM(d16:1-18:0) were increased ($p=0.04$). A number of phosphoethanolamines (PE) were

dysregulated but with no consistent pattern: PE (17:1-18:1); PE(18:0-18:1); PE(24:0-18:1), and PE(P-18:0-16:0) were decreased in IUGR hearts vs. CTRL, whereas PE(17:0-18:1) and PE(18:0-18:1) were increased. Lastly, cardiolipin (CL) (20:2/16:1/18:1/18:3) and phosphatidylinositol (PI) (18:0/18:1) were found in greater levels in IUGRs compared with CTRL (Supplemental Table 4).

Next, we analyzed the lipidomics data using the “Metabolomics Analysis” tool from IPA. As expected, we identified the “Lipid Metabolism and Transport” and the “Cardiovascular Disease” networks to be mostly affected by maternal undernutrition. When these two networks were merged (Figure 7A), several focus molecules were detected including peroxisome proliferator-activated receptor gamma (PPAR γ). The protein expression of PPAR γ showed a tendency to decrease in both male and female IUGRs vs. CTRLs, however, reached a statistical significance only in males (Figure 7B). In addition, female IUGRs had significantly higher levels of PPAR γ compared to males ($p=0.04$).

4. Discussion

This study yielded several important findings. In general, we found that fetal growth restriction resulted from maternal undernutrition is associated with significant sex-dependent changes in the fetal cardiac development at the epigenetic, molecular, and metabolic levels. First, there is a fetal sex-dependent accumulation of fibrotic tissue and dysregulation of fibrosis-related transcription factors in the hearts from IUGR baboon fetuses vs. controls. Second, fetal cardiac autophagy is activated in IUGR fetuses but, again, in a sex-dependent manner. Third, there is a sexually dimorphic expression of myocardial miRNAs in both control and IUGR baboons. Fourth, cardiovascular disease-related miRNAs were dysregulated in the hearts of male and female IUGR baboons. Fifth, we found changes in the fetal cardiac lipid profile as result of maternal undernutrition.

Human studies and several animal models show that calorie restriction is the most effective nutritional intervention to slow aging [77] and prevent chronic diseases [78]. Calorie restriction ameliorates hyperglycemia and hyperlipidemia [79], major risk factors for ischemic heart disease, attenuate atherosclerotic lesion formation [80], pathological cardiac hypertrophy [81], and ischemia-induced myocardial damage in animal models [82]. Calorie restriction during pregnancy, however, has the opposite effect. A strong correlation between inadequate nutrition of pregnant women and adverse offspring outcomes has been extensively demonstrated in the cardiovascular system [83] areas have mostly been studied in this context: the effect of maternal undernutrition on arterial blood pressure, endothelial function, and blood glucose homeostasis [84-86].

Sexual Dimorphism in the Fetal Response to Maternal Undernutrition

Baboon model of maternal undernutrition and other animal models of developmental programming [87] show that the adaptive response of offspring to an adverse maternal environment is strongly modulated by their sex with males being affected more than females. In this study, sex-related changes were found in the accumulation of fibrotic tissue, activation of autophagy, expression of cardiac miRNAs and transcription factors. Sexual dimorphism in expression of autophagy-related gene ATG7, pro-inflammatory transcription

factor NF κ B1, and nuclear receptors HNF4 α and PPAR γ has been observed in the control group. In addition, several genes responded to maternal undernutrition in a sex-dependent manner including NF κ B1, TGF β , and ALK1. Interestingly, however, no sexual dimorphism in cardiac lipid profiles was observed suggesting that similar metabolic patterns in male and female offspring could be achieved via different molecular and epigenetic perturbations. Differences in gene expression between males and females have been previously reported in liver[88] and brain[89]. Our team have recently shown sex differences in expression of genes associated with autophagy and lysosomal function in the placenta [90]. Interestingly, a study in the mouse implicates HNF4 α as transcription factor responsible for regulating genes that are preferentially expressed in each sex[91]. It seems that HNF4 α induces male-predominant expression in the liver in addition to suppressing the expression of other transcription factors that might have active role in the female [91]. In our study, female CTRL animals express less cardiac HNF4 α than males, which might at least partially explain sex differences in levels of miRNAs as well as metabolic, fibrotic and proinflammatory factors. Further experiments are needed to determine the role of HNF4 α in a sex-specific gene expression in the heart.

Sex-dependent responses to maternal undernutrition have been shown before. Sex-dependent changes in renal mitochondrial function were observed in the baboon model of maternal undernutrition [16]. Using a swine model of maternal undernutrition, Gonzalez-Bulnes et al. [92] showed that female conceptuses had higher viability rates and lower incidence of severe growth retardation. Differences in developmental patterns between male and female fetuses were evident and significant, indicating protective strategies for the adequate growth and postnatal survival of the females including the fact that both male and female fetuses prioritized brain development, but female fetuses were still able to maintain the development of other organs such as liver, intestine, and kidneys. In the rat model of maternal nutrient restriction, [93] male offspring showed severe hypertension and exhibited the highest increase in carbonyls whereas female offspring remained normotensive and did not exhibit differences in any of the oxidative stress biomarkers compared to controls.

Sex-specific differences in offspring outcomes from developmental programming models were also demonstrated in farm animals, mainly sheep [94-97] and cows [98]. A male offspring-predominant hypertensive effect, similar to that demonstrated in rat models, has been observed in sheep fed a low-methionine and vitamin B diet *in utero* [94]. Studies in sheep have also demonstrated that a nephrogenesis is impaired in male offspring exposed to nutrient restriction [95]. Catch-up growth was observed in young adult male cows when exposed to a low-protein diet in the third trimester, with no significant difference in females [98]. Thus, evidence from experimental models demonstrate a sexual dimorphism in the offspring's response to adverse *in utero* environment with males affected to a greater extent than females, a phenomenon that puts male offspring at higher risk of cardiovascular and metabolic diseases in later life [99]. It has been previously suggested that aging can abolish these sex differences implicating a loss of protection against programmed cardiovascular risk in females in later life [99]. Yan et al., however, have reported significant changes in expression of key glycolytic and mitochondrial proteins in the hearts of old males but not females in a primate model (*Macaca fascicularis*) of aging [100]. These changes are similar to those seen in the diabetic or failing hearts and could be implicated in the mechanism for

the cardiomyopathy of aging in male offspring. Further studies are needed to clarify the mechanisms that contribute to the development of cardiovascular diseases in IUGR individuals and the effect of sex across the lifespan on cardiovascular risk following a developmental insult in order to determine the clinical implications for these findings.

Four major factors could potentially cause sex differences in developmental programming. First, differences in patterns of development, i.e. genetic and transcriptional factors regulating the development from the earliest stages of preimplantation [101] and formation of placenta [102]. Second, the timing of development, for example, human male and female fetuses are known to develop at different rates [103], and the rate of growth may be critical, when the embryo is exposed to a given insult [104]. Third, exposure to sex steroids *in utero*, which has been shown to affect fetal and placental metabolism, for example, male fetuses are exposed to higher levels of androgen compared to female fetuses [105]. It has been also reported that sex differences in insulin sensitivity may be associated with the effect of estrogen as a leptin sensitizer on fetal brain [106]. And fourth, sex-specific differences in epigenetic modulations may be associated with different phenotypes in the animal models of developmental programming [107].

Sexual Dimorphism in Dysregulation of Fetal Cardiac miRNAs in Response to MNR

Cardiac miRNAs exhibit a developmental stage- and cell type-specific expression patterns, and also regulate distinct myocardial cellular processes such as proliferation, differentiation, cell metabolism, apoptosis, and angiogenesis [108]. The role of cardiac miRNAs in fetal programming has not been well-elucidated. We have recently shown a dysregulation of fetal cardiac miRNAs in the baboon model of a high fat diet-induced maternal obesity [35]. We designed this study to determine the role of three important aspects in the regulation of cardiac miRNA: the effect of maternal undernutrition, fetal growth restriction, and fetal sex. Surprisingly, we found no correlation between expression of miRNAs and the birth weight leaving maternal undernutrition as a sole cause of miRNA dysfunction in the IUGR group. We found sexual dimorphism in expression of cardiac miRNAs in both control and IUGR groups. We showed that the sex-specific regulation of cardiac miRNAs can be at least partially explained by different regulation of the upstream transcription factors such as HNF4 α and NF κ B1. Sex differences in the miRNA processing, maturation and transport could be another explanation for this phenomenon. The effect of estrogen on miRNA network has been previously reported by Queiros et al. [109] the healthy and hypertrophied mouse hearts. The effect of estrogen on fetal miRNA expression, however, remains unclear.

Myocardial Fibrosis in the Offspring of Maternal Undernutrition

In the present study, we found an increase in cardiac fibrosis in male but not female IUGR baboons. Myocardial fibrosis has been already shown in the offspring of adverse pregnancies. We have previously reported cardiac fibrosis in the baboon fetuses, exposed *in utero* to maternal obesity. In sheep studies of maternal obesity, an accumulation of collagen was also reported [110]. Nguyen et al. [111] found cardiac fibrosis in the adult offspring exposed to chronic alcohol consumption during gestation. The presence of cardiac fibrosis is an important determinant of abnormal myocardial stiffness that contributes to diastolic

dysfunction [112]. In fact, our recent MRI measurements showed a substantial diastolic dysfunction in 5-9-year-old baboons, offspring of one of our IUGR cohorts [113].

Transforming growth factor beta (TGF β) is a cytokine that regulates both cardiomyocyte and fibroblast biology and its expression and signaling activity are increased during cardiac remodeling [114]. In cardiac fibroblasts, binding of TGF β to its receptors results in the activation of the canonical signaling pathway, which comprises activation of SMADs, which translocate to the nucleus and drive the expression of TGF β -responsive genes [114]. In cardiomyocytes, binding of TGF β to its receptors leads to the activation of a non-canonical pathway involving the phosphorylation of TGF β -activated-kinase 1 and p38 [115]. Activation of the noncanonical TGF β -pathway in myocytes during cardiac remodeling has been shown to regulate the development of myocyte hypertrophy [116].

Interestingly, despite the accumulation of fibrotic tissue in the hearts of male IUGR fetuses, the expression levels of TGF β , ALK1, and SMAD3 were decreased. Suppression of TGF β signaling in fetal hearts has been previously reported in the mouse model of gestational diabetes [117]. Our observation might be explained by a mixed cell population of fibroblasts and cardiomyocytes in our samples. Unfortunately, the limited tissue availability from the present study did not allow for cell-specific localization of TGF β by immunohistochemistry. Another potential explanation could be the fact that most members of the TGF β pathway are known to be targeted by one or more miRNAs [118]. The reduction in TGF β signaling in male IUGRs could represent a secondary effect of an increased expression of several upstream miRNAs including miR-20a/b, miR-27b, and miR-130 (Figure 4). Undoubtedly, further work is needed to thoroughly investigate these observations.

Activation of Cardiac Autophagy in Response to Maternal Undernutrition

Autophagy is responsible for the turnover of long-lived proteins and of intracellular structures that are damaged or functionally redundant [119-121]. The important role for autophagy at early neonatal stages has been previously reported [122]. Formation of autophagosomes has been identified by electron microscopy in different neonatal tissues, including kidney [123], liver [124] skeletal muscle, [125] and heart [126]. Soon after birth, mammals encounter the first, and probably the most severe period of starvation during their lifespan associated with the abrupt interruption of the transplacental nutrient supply [127]. It has been shown that neonates adapt to this adverse circumstance by inducing autophagy. The importance of autophagy for neonatal survival was clearly demonstrated by targeted inactivation of the autophagy-related genes Atg5 and Atg7. [128, 129] Mice deficient in these genes were apparently normal at birth but died within one day after birth. Three additional potential explanations for activation of autophagy in neonatal tissues were proposed including 1) glycogen degradation [124-126, 130]; 2) rapid reprogramming of cell metabolism during the fetal-to-neonatal transition, requiring not only the upregulation of new proteins but also the elimination of embryonic constituents [131-133]; and 3) the response to oxidative stress at birth [134]. Here we observed evidence of increased fetal cardiac autophagy in male IUGRs. It is unclear whether maternal nutrient restriction leads to the early appearance of neonatal cardiac autophagy or the existing cardiac autophagy has

been enhanced in the IUGR animals. Also, it remains to be shown if increased autophagy will eventually result in adult cardiac dysfunction in these animals.

Lipidomic Adaptation in Fetal Cardiac Tissue in Response to MNR

The human fetus adapts to maternal undernutrition with certain physiological and metabolic changes, including a slowing of growth, redistribution of blood flow, and changes in the production of hormones [135]. Lipids play a significant role in the cardiac adaptation to environmental and genetic influences. Dysfunctional cardiac lipid metabolism has been previously linked to inflammation and oxidative stress [136, 137] greatly increasing the risk for atherosclerotic and cardiovascular diseases [138]. Using lipidomics approach, we showed that lipid content of fetal hearts could be dramatically modified by maternal undernutrition. There was a substantial remodeling of the diglyceride (DG) pool with a significant increase in some very long chain DG (18:0-22:6, 18:1-20:4, 18:2-20:0). The myocardial TG pool, in contrast, was decreased dramatically in IUGR baboons vs. controls. It seems likely that undernutrition pushes the balance between TGs and DGs towards DG formation because fatty acid chains are being catabolized from the TGs to balance out the energy deficit. Another potential explanation could be an elevation in glucose oxidation causing a reduction in myocardial TG levels since glucose provides the glycerol backbone required for TG synthesis. In fact, increased glucose uptake has been demonstrated in the fetal hearts of sheep exposed to nutrient deprivation from placental insufficiency, which appear to be an adaptive process to maintain myocardial energy supply and support myocardial function and growth [139]. Increased adipocyte hypertrophy previously reported in the IUGR animals [140, 141] could be another potential explanation of reduced TG. The accumulation of unused fatty acids in nearby adipocytes may protect the myocardium from lipotoxicity. On the other hand, large prospective cohort studies have demonstrated that the increased adipocyte volume predicts the future likelihood of incident cardiovascular disease and major cardiovascular events [142].

So far, nothing is known about the role of plasmalogens in fetal hearts, except for the fact that fetal plasmalogen content differs from maternal: fetal heart contained more ethanolamine plasmalogen than choline plasmalogen, whereas in adult myocardial tissue the concentration of these was reversed [143]. Our study found a significant increase in the myocardial content of phosphatidylethanolamine plasmalogens in the IUGR fetuses compared to controls. Several biological functions have been attributed to plasmalogens including endogenous antioxidants [144], mediators of membrane dynamics [145], and storages of polyunsaturated fatty acids (PUFAs) [146] and lipid mediators [147]. The physiological role(s) of plasmalogens in fetal hearts in association with different maternal environments remains elusive; however, an increase in their levels in response to maternal undernutrition may be a physiological adaptation to the limited nutrient availability.

In summary, the present study suggests that fetal hearts adapts to maternal nutrient restriction in a sex-dependent manner. We observed myocardial perturbations on the morphometric, epigenetic, molecular, and metabolic levels. If these changes persist into adult life, they will eventually lead to the development of cardiovascular diseases. We believe that this study increases our knowledge about the role of maternal environment and

fetal sex in heart development and potential determination of adult cardiac phenotype, which may be of translational value for subsequent studies in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALK1	activin receptor-like kinase-1
ATG7	autophagy-related protein-7
CL	cardiolipin
CTGF	connective tissue growth factor
CDX2	caudal type homeo box transcription factor 2
DG	diglycerides
HNF4α	hepatocyte nuclear factor 4 alpha
IUGR	intrauterine growth restriction
LC3B	microtubule-associated protein 1B-light chain 3
MNR	maternal nutrient restriction
NFκB1	nuclear factor kappa B subunit 1
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PPARγ	peroxisome proliferator-activated receptor gamma
SM	sphingomyelins
TSP-1	Thrombospondin-1
TGFβ	transforming growth factor - β
TG	triglycerides

Highlights

- Maternal undernutrition causes cardiac fibrosis and activates cardiac autophagy in a sex-dependent manner;
- The expression of fetal cardiac miRNA is dysregulated by maternal undernutrition in a sex-dependent manner
- Maternal undernutrition affects fetal cardiac lipid profile.

Figure 1. Histologic characterization of fetal hearts from male and female CTRL and IUGR baboons on day 165 of gestation

A. Representative H&E and Trichrome staining; **B.** correlation curves between the accumulation of fibrotic tissue and the birth weight; $n=6$ in each experimental group, $p<0.05$.

Figure 2. Changes in protein expression of fibrotic factors in fetal hearts following maternal undernutrition

A, Representative images, and **B-F**, Quantification of protein levels of the major fibrosis markers evaluated using specific antibodies for TSP1 (**B**); CTGF (**C**); SMAD3 (**D**); TGFβ (**E**), and ALK-1 (**F**). Data were normalized to β-actin. Data presented as box-and-whisker plot, the bottom, and top of the boxes indicate the 25th and 75th percentiles, and the whiskers indicate the 5th and 95th percentiles, × marks outliers, n=6-7 in each experimental group, *, p<0.05 vs CTRL.

Figure 3. Maternal undernutrition activates cardiac autophagy in a fetal sex-dependent manner
A. Representative images, **and B-C.** Quantification of Western Blots for autophagy marker ATG7 (**B**) and the ratio of LC3B-II to LC3B-I (**C**) in total myocardial protein. Data were normalized to β -actin. Data presented as box-and-whisker plot, the bottom and top of the boxes indicate the 25th and 75th percentiles, and the whiskers indicate the 5th and 95th percentiles, $\times$ marks outliers, $n=6-7$ in each experimental group, *, $p<0.05$ vs. CTRL, #, $p<0.05$ vs. males within the same experimental group.

Figure 4. Heat maps depicting unsupervised hierarchical clustering of normalized expression for known and novel (PC) cardiac miRNAs in male (left) and female (right) CTRL and IUGR baboons

Each column depicts miRNA expression from one baboon heart. Green designates low expression and red designates high expression relative to mean levels across all samples for each miRNA, $p < 0.05$.

Figure 5. The impact of sex on fetal cardiac miRNA expression in the CTRL and IUGR groups
 Ratio male/female of known miRNAs within CTRL (A) or IUGR (B) groups. Representative images (C) and quantification graphs for protein levels of transcription factors HNF4α (D); NFκB1 (E), and CDX2 (F). Data were normalized to β-actin. Data presented as box-and-whisker plot, the bottom and top of the boxes indicate the 25th and 75th percentiles, and the whiskers indicate the 5th and 95th percentiles, × marks outliers, n=6-7/group, #, p<0.05 vs. males within the same experimental group, *, p<0.05 vs. CTRL.

Figure 6. Perturbations in fetal cardiac lipid metabolism as result of maternal undernutrition
 Box plots demonstrate the range of values for each detected metabolite in CTRL (light gray) and IUGR (dark gray) groups. **A.** Diglycerides; **B.** Triglycerides; **C.** Short phosphatidylcholines, **D.** Long phosphatidylcholines, and **E.** Plasmalogens. n=6 in each experimental group, *, p<0.05 vs. CTRL.

Figure 7. Analysis of up- and down-regulated fetal cardiac metabolites in IUGR animals using Ingenuity Pathways Analysis (IPA)

Metabolites highlighted in green (downregulated) and red (upregulated) shading were identified by mass spec analysis. Metabolites and/or proteins highlighted in light brown were not identified but were linked to the identified proteins/metabolites by examination of the IPA curated database. Lines connecting the molecules indicate molecular relationships. Dashed lines indicate indirect interactions and solid lines indicate direct interactions. B. Western blots for cardiac PPAR γ in CTRL and IUGR baboon fetuses. Data were normalized to β -actin. Data presented as box-and-whisker plot, the bottom and top of the boxes indicate the 25th and 75th percentiles, and the whiskers indicate the 5th and 95th percentiles, $\times$ marks outliers, $n=6-7$ /group, *, $p<0.05$ vs. CTRL, #, $p<0.05$ vs. males within the same experimental group.

Table 1

Effect of maternal undernutrition on fetal morphometric parameters at cesarean section (0.9 gestation).

Groups	Males (n=6)		Females (n=7)	
	CTRL n=3	IUGR n=3	CTRL n=4	IUGR n=3
Maternal Age (years)	10±1.50	10.5±0.10	9.6±1.29	12.8±0.42
Maternal Weight at C-section (kg)	15.9±0.98	14.8±1.43	17.2±0.98	14.6±1.11 *
Fetal Measures				
Fetal Weight (g)	784±46	715±55	714±42	626±27 *
Placental Weight (g)	180±10.00	162±1.22 *	175±12.60	142±5.78 *#
Brain (g)	82.3±4.44	78.6±4.84	73.3±3.42#	77.8±2.62
Heart (g)	4.6±0.38	3.9±0.39	4.3±0.63	3.5±0.09#
Kidney (g)	3.8±0.52	3.7±0.15	3.2±0.11	3.5±0.59 *#
Liver (g)	20.0±0.70	19.1±2.70	20.8±0.59	17.2±0.61 *
Lung (g)	16.5±1.45	21.9±1.46	18.8±0.87#	17.4±1.16 *
Pancreas (g)	0.4±0.06	0.5±0.10	0.6±0.06	0.6±0.16
Spleen (g)	1.5±0.26	1.3±0.24	1.3±0.15	1.1±0.02
Thymus (g)	3.5±0.88	2.5±0.09	3.5±0.76	2.5±0.60
Fetal Measures as Percent Fetal Body Weight				
Placenta	23.1±1.15	23.2±2.36	24.6±1.91	22.8±1.11
Brain	10.6±1.14	11.0±0.17	10.3±0.36	12.4±0.37#
Heart	0.6±0.04	0.6±0.05	0.6±0.05	0.6±0.03
Kidney	0.5±0.05	0.5±0.04	0.5±0.03	0.6±0.11
Liver	2.6±0.10	2.7±0.19	2.9±0.09	2.8±0.19
Lung	2.1±0.09	3.1±0.15 *	2.6±0.09#	2.8±0.23
Pancreas	0.1±0.00	0.08±0.00	0.1±0.1	0.1±0.03
Spleen	0.2±0.00	0.18±0.03	0.2±0.02	0.2±0.01
Thymus	0.4±0.09	0.33±0.00	0.5±0.07	0.4±0.08

CTRL, control group; IUGR, intrauterine growth restricted fetuses; data presented as mean ±SD,

* p<0.05 vs. CTRL group;

p<0.05 females vs. males within the same group.