Published in final edited form as:

Int J Obes (Lond). 2020 July ; 44(7): 1497–1507. doi:10.1038/s41366-019-0485-y.

Gestational diabetes and maternal obesity are associated with sex-specific changes in miRNA and target gene expression in the fetus

Apoorva Joshi, MS[1], **Rikka Azuma, BA**[1], **Rita Akumuo, MPH**[1], **Laura Goetzl, MD**[2,3], **Sara E. Pinney, MD, MS**[1,4,5,6]

[1]Division of Endocrinology and Diabetes, Children's Hospital of Philadelphia, Philadelphia, PA USA

[2]Department of Obstetrics and Gynecology, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA

[3]Department of Obstetrics, Gynecology and Reproductive Sciences, McGovern School of Medicine, University of Texas, Health Sciences Center at Houston, Houston, TX, USA

[4]Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

[5]Center for Research in Reproduction and Women's Health, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

[6]Center of Excellence in Environmental Toxicology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

Abstract

Background/Objective—Pregnancies complicated by gestational diabetes (GDM) or maternal obesity have been linked to the development of diabetes, obesity and fatty liver disease later in life with sex-specific manifestations. Alterations in miRNA expression in offspring exposed to GDM and maternal obesity and effects on hepatic development are unknown. Here we describe how exposure to maternal obesity *in utero* leads to sex-specific changes in miRNA and target gene expression in human fetal liver.

Methods—Candidate miRNA expression was measured in 2nd trimester amniotic fluid (AF) from women with GDM. Targets of differentially expressed miRNAs were determined and pathway enrichment of target genes was performed. MiRNA and target gene expression were measured in a separate cohort of 2nd trimester primary human fetal hepatocytes (PHFH) exposed to maternal obesity via QPCR and western blot. All studies were IRB approved.

Corresponding Author: Sara E. Pinney, MD, MS, Division of Endocrinology and Diabetes, Children's Hospital of Philadelphia, Abramson Research Building, Room 510B, 3615 Civic Center Boulevard, Philadelphia, PA 19104. pinneys@email.chop.edu Phone: 267-426-3902 Fax: 215-590-3053.

Results—GDM exposed AF had significant increases in miRNAs 199a-3p, 503-5p, and 1268a (fold change (FC) ≥1.5, p<0.05). Female offspring specific analysis showed enrichment in miRNAs 378a-3p, 885-5p, and 7-1-3p (p<0.05). MiRNA gene targets were enriched in hepatic pathways. Key genes regulating *de novo* lipogenesis were upregulated in obesity exposed PHFH, especially in males. Significantly altered miRNAs in GDM AF were measured in obese exposed PHFH, with consistent increases in miRNAs 885-5p, 199-3p, 503-5p, 1268a and 7-1-3p (FC ≥1.5, p<0.05). Female PHFH exposed to maternal obesity had increased expression of miR-885-5p, miR-199-3p, miR-503-5p, miR-1268s and miR-7-1-3p, (p<0.05), corresponding to decreased target genes expression for *ABCA1, PAK4* and *INSR*. In male PHFHs, no miRNA changes were measured but there was increased expression of *ABCA1, PAK4*, and *INSR* (p<0.05).

Conclusion—Our data suggest sex-specific changes in miRNA and gene expression in PHFH may be one mechanism contributing to the sexual dimorphism of metabolic disease in offspring exposed to GDM and maternal obesity *in utero.*

Introduction

Gestational diabetes (GDM) and maternal obesity have profound effects on the intrauterine metabolic milieu, induce marked abnormalities in glucose homeostasis and insulin secretion in the fetus and are linked to obesity, diabetes and non-alcoholic fatty liver disease in the offspring[1–6]. Epidemiologic and animal studies support the concept that there is a critical window of developmental programming during which *in utero* exposures are associated with an increased susceptibility to obesity and diabetes [7, 8] but the molecular mechanisms underlying this phenomenon are unknown. Alterations in epigenetic modifications, including DNA methylation and histone modifications are proposed as a mechanism by which an *in utero* exposure can lead to permanent changes in cellular function and ultimately metabolic disease later in life [9].

MicroRNAs comprise a large family of small non-coding RNAs and have emerged as key regulators of metabolic homeostasis, but the role that miRNAs play in fetal development is not well understood. Recent technology has enabled the discovery of circulating miRNAs, which can function as signaling molecules and disease biomarkers. In relation to pregnancy, miRNAs are abundant in maternal plasma, amniotic fluid (AF) and placenta and are involved in the proliferation and differentiation of trophoblast cells and immunological defense[10–13]. Furthermore, abnormalities in miRNA processing are associated with poor placental function and failed embryonic development[11].

Although placental dysfunction is a well-known contributor to fetal growth, little is known about the origin of miRNAs identified in AF or whether placental derived miRNAs enter the fetal compartment. At 16–18 weeks gestational age (GA), AF is comprised of mostly fetal-derived components given that fetal skin is not fully keratinized and fetal urine does not make a substantial contribution until after 20 weeks GA[14]. Therefore it is hypothesized that many of the miRNAs measured in AF are fetal derived and thus alterations in miRNA expression in AF from women with GDM may provide insight into the mechanisms by which GDM affects the developing fetus.

Given the potential role that miRNAs play in fetal development, we investigated whether expression of circulating miRNAs in AF collected at GA 16–18 weeks is altered in women who were later clinically diagnosed with GDM between 24–28 weeks GA. In addition, we sought to determine whether changes in miRNA levels from women with GDM or maternal obesity were associated with abnormal fetal liver development.

Materials and Methods

Study Population

Second trimester amniotic fluid samples—AF samples were collected from women with healthy singleton term pregnancies without maternal health conditions, pregnancy complications, or fetal anomalies. AF specimens were collected from women undergoing amniocentesis at GA 16–18 weeks from 2002–2006 and stored in polypropylene cryogenic vials at −80°C adhering to a strict research protocol[15]. Data abstracted from reproductive genetics charts and post-birth outcome surveys included maternal age, race and ethnicity, GA at amniocentesis, indication for amniocentesis, cytogenetic testing results, sex of offspring and pregnancy outcome data including birth weight, GA at birth, and maternal health history, including complications encountered throughout the duration of the pregnancy. Samples were collected after written informed consent was obtained under a research protocol approved by the University of Pennsylvania and the Children's Hospital of Philadelphia.

For the present study, we used a nested case control design selecting 20 AF samples from mothers subsequently diagnosed with GDM and 20 control AF samples with no history of maternal GDM. Samples were matched 1:1 for maternal age, gestational age at amniocentesis, maternal race/ethnicity and offspring sex. The most common indication for amniocentesis was advanced maternal age (> 35 years). GDM status was identified through an outcome survey completed one month after delivery and confirmed by measurement of AF c-peptide concentrations >4-fold control as previously reported[16]. Clinical GDM testing is typically performed between 24–28 weeks GA. No data on GDM treatment was collected.

Primary Human Fetal Hepatocytes (PHFH)—Fetal liver was obtained in accordance with an approved institutional review board protocol. Tissue obtained from legally aborted second trimester fetuses between 17–19 weeks gestation from women with BMI> 30 or normal weight women (BMI ≤ 25). Liver was minced in Seglan's buffer containing 3 mg/mL collagenase I (Roche, Indianapolis, IN) and digested for 30 minutes at 37°C. PHFH cultures were derived following a previously published protocol[17]. Cells were collected by centrifugation, resuspended in William's media (Gibco, Gaithersburg, MD) and passed through a 21-gauge needle to obtain a single cell suspension. Cells were washed and seeded in 6 well collagen coated plates with Williams media supplemented with nicotinamide (10mmol/L), Hepes (20mmol/L), $NaHCO_3$(17 mmol/L), pyruvate (550 mg/L), ascorbic acid-2-phosphate (0.2 mmol/L), glucose (14 mmol/L), glutamine (2mmol/L) and 10^{-7} mol/L dexamethasone, ITS+premix containing insulin (6.25 μg/mL), transferrin (6.25 μg/mL) selenious acid (6/25 ng/mL) bovine serum albumin (1.25 ng/mL) and linoleic acid (5.35 μ/mL) antibiotics and 5% fetal bovine serum. After 4 hours the media was changed to

remove serum and epidermal growth factor was added (20 ng/mL). All cultures were maintained at 37°C in 5% CO_2 with medium changed every 2 days. Experiments were performed at 90% confluence prior to passaging cells.

Multiplex Circulating miRNA Assay

Eighty-eight miRNA candidates were generated from a PubMed literature review with search terms including "miRNA" and "Type I and Type 2 diabetes, GDM, preeclampsia, adipogenesis, obesity, and nonalcoholic fatty liver disease" [13, 18–33] (Supplemental Table 1). Individual candidate miRNA abundance was measured via Multiplex Circulating miRNA assay (Abcam, FirePlex, Cambridge, MA). Samples were digested and hybridized to miRNA specific hydrogel particles with a universal biotinylated adapter labeled with a fluorescent reporter, and quantified with EMD Millipore Guava 8HT flow cytometer. Positive and negative controls were included to reduce inter-plate and inter-well variability. MiRNA spike-in target probes measured hybridization success. Blank hydrogel particles were run to define background fluorescence. Abcam FirePlex Analysis Workbench software was used for data analysis (https://www.abcam.com/kits/multiplex-immunoassays-firefly-analysis-workbench-software). Normalization was performed via geNorm algorithm using the three most stable miRNAs across all samples (hsa-let-7d-5p, hsa-mir-107, and hsa-mir-342-3p) [3435]. Data was log converted to eliminate directional bias. Geometric mean and fold changes were calculated for each miRNA based on normalized expression data.

Prediction of miRNA Targets and Pathway Analysis

TargetScan was used to identify potential gene targets of differentially expressed miRNAs through analysis of seed binding sequence sites [36]. Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) identified significantly enriched canonical and toxicological pathways for gene targets of differentially expressed miRNAs.

Total miRNA from PHFH was isolated using miRNeasy (Qiagen). MiRNA expression was measured using qPCR SYBR Green (Qiagen) and normalized with miR-16, selected due to minimal change in CT values across all samples. Fold change was calculated and plotted as mean ± SEM (GraphPad Prism).

Target Gene mRNA and Protein Expression

Total RNA was isolated from PHFH using RNeasy (Qiagen). Complementary DNA was prepared using Invitrogen's SuperScript IV Vilo master mix and qPCR with Taqman probes for *ABCA1, PAK4,* and *INSR* using *ACTB* and *GAPDH* as housekeeping genes. Protein was extracted PHFH and quantified using Pierce BCA assay (Thermo Fisher, Waltham, MA). Samples were run on 4–12% bis-tris gels and transferred on nitrocellulose membrane using iBlot2. Antibodies used were PAK4 (1:500, Cell Signaling, Danvers, MA), INSR (Invitrogen, ThermoFisher, 1:1000), β-actin (1:1000, Sigma Aldrich, St. Louis, MO) and secondary antibody (1:10000, Licor, Lincoln, NE).

Statistical Analysis

Two sample t-tests or Mann-Whitney tests were used to compare means between 2 groups. Due to previous studies showing offspring sex as an important variable in fetal

programming, separate analyses based on the sex of the offspring were performed. Benjamini Hochberg adjustment for multiple hypothesis testing was applied for the AF circulating miRNA assay, although no candidate miRNAs reached statistical significance using $q<0.05$. Therefore for the AF circulating miRNA assay, fold change>1.5 or <0.6 and raw $p<0.05$ was considered statistically significant. For QPCR and western blot studies, $p<0.05$ was considered significant. SAS 7.0, GraphPad Prism 7.0 and Firefly Analysis Workbench (Abcam, Cambridge, UK) were used to perform the statistical analyses.

Results

Table 1 describes the maternal and infant characteristics of the AF samples used for the circulating miRNA assay. Due to the matching algorithms used for the nested case-control design, there were no differences in maternal race, ethnicity, or GA of amniocentesis, or GA at birth (Table 1). The vast majority of the samples were collected from white, non-Hispanic women. The mean age of all women was 37.4 ± 3.22 years and the most common indication for amniocentesis was due to advanced maternal age. All AF samples studied were collected at a mean GA of 16.2 ± 0.56 weeks and the mean GA at birth was 39.0 ± 1.47 weeks. There were no statistical differences in infant birth weight, indicating that the GDM was well controlled during pregnancy.

Multiplex Circulating miRNA Assay Results

Eighteen miRNAs had significant changes in expression in GDM AF samples compared to controls with $p<0.05$ but only 6 also met criteria of FC ≥ 1.5 or ≤ 0.66 (Table 2). In the analysis of all 40 AF samples (20 GDM/20 control), miRNAs hsa-miR-199a-3p, hsa-miR-503-5p, and hsa-miR-1268a were increased in AF of women with GDM (fold change: 1.77, 1.5, 1.77 respectively; FC ≥1.5, p ≤0.05). When the analysis was limited to AF samples from female offspring (n=20; 10 GDM /10 control), GDM samples had significant increases in hsa-miR-378a-3p, hsa-miR-885-5p, and hsa-miR-7-1-3p (FC ≥1.5, $p<0.05$). When only male offspring samples were analyzed (10 GDM/10 Control), GDM samples again showed a significant increase in hsa-miR-199a-3p (FC ≥1.5, $p<0.05$).

Pathway Enrichment Results

IPA was performed to determine significantly enriched canonical pathways based on mRNA targets of the differentially expressed miRNAs (Supplemental Tables 2–4). For all samples, pathway analysis identified 161 significantly enriched canonical pathways ($p<0.05$), 32 for female offspring, and 88 for male offspring. Across all 3 analyses, significantly enriched canonical pathways included cell growth and cell cycle regulation, inflammation, stem cell development and there was significant crossover between enriched canonical pathways for each analysis. Similarly, IPA was used to determine enriched toxicological pathways (Supplemental Table 5) and showed consistent enrichment in liver-based processes including proliferation and apoptosis of hepatocytes and hepatocellular carcinoma pathways.

We did not have access to additional AF cohorts exposed to GDM in order to validate our results of the circulating miRNA assay. Therefore, given the strong enrichment of liver related pathways in the IPA toxicological pathway analysis, we hypothesized that miRNAs

enriched in AF of women with GDM may originate in fetal liver and that PHFH exposed to maternal obesity may also be enriched for these miRNA species. Although we did not have BMI data for the women in the GDM cohort, the vast majority of women with advanced maternal age and GDM are obese. Furthermore, the mothers with obesity from which the PHFH were obtained had not completed GDM testing since it is typically performed at 24–28 weeks GA. Although we acknowledge that GDM and maternal obesity are distinct physiological processes, these conditions have considerable overlap in effects on fetal development. In addition, we found that PHFH exposed to maternal obesity had increased expression of key genes regulating *de novo* lipogenesis, supporting the concept that maternal obesity has profound effects on fetal liver metabolism and the effect was more prominent in PHFC from male offspring (Figure 1). Therefore we measured the differentially expressed miRNAs identified from the GDM exposed AF and their physiologically relevant target gene expression in second trimester PHFH exposed to maternal obesity in an effort to determine whether changes in miRNA expression and effects on target gene expression may be one mechanism by which an altered intrauterine milieu affects fetal development.

Confirmation of miRNA enrichment and target gene expression in second trimester PHFH exposed to maternal obesity

Clinical characteristics of the source subjects for PHFH samples are shown in Table 3. MiRNAs enriched in GDM AF were measured in second trimester PHFHs exposed to maternal obesity (Figure 2). MiRNA-885-5p expression was significantly increased in maternal obesity exposed PHFH in analyses from all (FC: 2.75, $p<0.05$) and female only (FC: 3.58, $p<0,05$) samples (Figure 2a). MiR-199a-3p expression was increased in female PHFH exposed to maternal obesity (FC: 2.02, $p<0.05$) (Figure 2b) but no significant differences were measured when analyzing all samples together or in male samples. miR-503-5p was increased in female (FC: 2.81, $p<0.05$) and all PHFH (FC: 1.69, $p<0.05$) but not in male PHFH (Figure 2c) exposed to maternal obesity. MiR-1268a was increased in only female obesity exposed PHFH (FC: 3.7, $p<0.05$). miR-7-1-3p was increased in all (FC: 1.75, $p<0.05$) and female obesity exposed PHFH (FC: 2.63, $p<0.05$) but not males.

Using TargetScan, we found that several of the *de novo* lipogenesis genes in Figure 1 are targets of the differentially expressed miRNAs listed in Table 2; *FFAR4, PGC1A, SCD and SREBF1* are targets of miR7-3p, *SCD* is a target of miR-199-3p, and miR885-5p is a target of *EHHADH*. In the miRNAs and target genes listed above, there is a consistent pattern of increased expression of the miRNA in female PHFH but no change in miRNA expression male PHFH, corresponding to a greater increase in mRNA expression of the target gene in male PHFH compared to female PHFH. Therefore we performed additional experiments to determine if the sex specific changes in miRNA expression corresponded to changes in mRNA expression of additional target genes, selecting *ABCA1, PAK4 and INSR* as targets of miRNAs 885-5p, 199a-3p and 503-5p respectively. There were no significant changes in *ABCA1* mRNA expression in the all or female sample analyses but *ABCA1* mRNA expression was significantly increased in male PHFH (FC: 3.64, $p<0.05$) where no enrichment of miR-885-5p was measured (Figure 3a). *ABCA1* is a regulator of reverse cholesterol uptake in the liver and placenta [37]. *PAK4* is a target of miR-199a-3p involved in hepatocyte proliferation and growth[38]. No significant changes were measured in *PAK4*

mRNA expression (Figure 3b) but PAK4 protein levels were significantly decreased in female PHFH exposed to maternal obesity (FC: 0.38, $p<0.05$) and significantly increased in male samples (FC: 3.16, $p<0.05$) (Figure 2c). MRNA expression of *INSR*, a target genes of miR-503-5p and an important regulator of fetal growth and metabolism, was decreased in female PHFH (FC: 0.65, $p<0.05$) and enriched in male PHFH (FC: 1.86, $p<0.05$) (Figure 3c). Protein levels of INSR trended higher in both male and female PHFH exposed to maternal obesity but did not reach statistical significance ($p=0.08$ and $p=0.05$, respectively) (Figure 3e).

Discussion

To our knowledge, this is the first report of significant changes in miRNA expression in second trimester AF from women who were diagnosed with GDM at 24–28 weeks GA. Although there is limited utility using AF as a biomarker to predict GDM, the enrichment of miRNAs in AF prior to the diagnosis of GDM strongly suggests that altered miRNA expression is a component of the intrauterine milieu in GDM pregnancies and either contributes to maternal metabolic abnormalities or affects the developing fetus much earlier in gestation than clinical GDM testing is currently performed. There is limited information about how miRNAs detected in AF alter fetal development, but several studies measuring miRNAs in serum of women with preeclampsia or diabetes suggest that alterations in miRNAs may impact offspring growth[13, 19, 31, 33].

A second major finding in our study is that miRNA and mRNA expression is dependent on offspring sex in offspring of women with GDM or maternal obesity. Sex specific differences in miRNA expression were noted in both AF and PHFH but the mechanisms contributing to this phenomenon remain unknown. Both epidemiological and animal studies based on the developmental origins of health and disease (DOHaD) hypothesis report that the effects of GDM and maternal obesity on the health of the offspring are sex-specific [39–41]. Work in this field is based on the premise that the gestational period is characterized by rapid cellular differentiation and growth and represents a critical window of exposure during which an altered intrauterine environment characterized by GDM or maternal obesity can lead to permanent changes in cellular function. Here we show that mRNA expression of key gene regulating hepatic *de novo* lipogenesis are increased to a greater degree in male PHFH exposed to maternal obesity than female obese exposed PHFH. Previous work from our group has shown sex-specific alterations in DNA methylation and gene expression in term placenta exposed to diabetes in pregnancy as well as sex-specific changes in metabolomics analysis from second trimester AF from women subsequently diagnosed with GDM [16, 42]. However, the mechanisms responsible for the sex-specific alterations have not been identified.

Since we did not have access to a separate cohort of second trimester AF samples from women with GDM, and based on the hepatic pathway enrichment of the miRNA target genes from the IPA analysis, we hypothesized that the differentially expressed miRNAs from GDM AF may be derived from or have critical functions in the developing liver. Although we acknowledge that maternal obesity and maternal GDM may have distinct programming effects on the fetus, there is likely much overlap in the maternal phenotypes during second

trimester of gestation. Although clinical testing for GDM is not typically performed before GA 24 weeks, we propose that the metabolic pathways affecting fetal development in obese women and those subsequently diagnosed with GDM have significant overlap in the second trimester of gestation.

We identified three miRNAs with significantly increased expression in GDM exposed AF in the analysis of all samples: miR-199a-39, miR-503-5p and miR-1268a. MiR-199a-3p is important to pancreatic beta cell function, hepatocyte growth and differentiation and the development and function of brown and beige adipocytes [18, 22, 43]. In a model of type 2 diabetes induced by a low protein maternal diet, adult offspring with glucose intolerance had increased miR-199a-3p in pancreatic beta cells leading to decreased mTOR signaling [18], an important pathway regulating cell growth and energy metabolism [18]. Inhibiting expression of miR-199a restores mTOR signaling and normalizes insulin secretion [18]. Studies suggest that placental mTOR signaling is an important sensing mechanism of maternal nutrient availability and fetal growth [44, 45]. Increased miR-199a-3p is associated with conditions induced by oxidative stress including steatohepatitis through regulation of the PAK4/MEK/ERK pathway at the protein level [38, 46]. We found that increased miR-199-3p is associated with decreased PAK4 protein expression in female PHFH exposed to maternal obesity but in male obese-exposed PHFH there was increased PAK4 protein expression but no enrichment of miR-199a-3p (Figure 2).

There is limited information describing how miR-503 affects fetal development but gene targets of miR-503 include *CCNE1*, *CDC25A*, and *CCND2*, all of which are involved in cell cycle regulation. Increased concentrations of circulating miR-503 were found in plasma of diabetic patients and in myocardial microvascular endothelial cells and skeletal muscle tissue in an animal model of type 2 diabetes [47–49]. Gene targets of miR-503-5p include *FGF2, VEGFA* and both *IGF1R* and *INSR* due to their sequence homology. Overexpression of miR-503-5p is associated with decreased *IGF1R* expression [50]. Infants born to mothers with GDM and maternal obesity may have macrosomia in part due to increased fetal insulin production acting as a fetal growth factor and second trimester AF from women diagnosed with GDM have been reported to have a 4–5 fold increase in fetal c-peptide concentrations [16]. For this reason, we hypothesized that exposure to maternal obesity may lead to changes in *INSR* expression in PHFHs. We found that expression of miR-503-5p was significantly increased and *INSR* mRNA levels were decreased in female PHFH exposed to maternal obesity, but in male PHFH, there was no change miR-503-5p expression while *INSR* levels were significantly increased (Figure 3d). However, we found a discrepancy between *INSR* mRNA expression and INSR protein levels, which were increased in both male and female PHFH exposed to maternal obesity, although this did not reach statistical significance (Figure 3e). It is possible that increased miR-503-5p corresponding to decreased *INSR* mRNA in female PHFH exposed to maternal obesity could represent a negative feedback response, functioning ultimately to dampen the increased INSR protein expression induced by maternal obesity. However, these relationships are not clear at this time and will require additional experiments in the future.

MiR-1268a was the final miRNA increased in the combined male and female GDM AF [19]. MiR-1268a interacts with *SLC8A8*, a plasma membrane transporter of creatine, and

B4FALNT3, a gene involved in structural modifications of cell-surface N-glycans, which regulate the metabolic function of the developing fetus[19].

When our analyses were limited to AF samples with female offspring, hsa-miR-378a-3p, hsa-miR-7-1-3p and hsa-miR-885-5p had significant increases in expression in GDM exposed AF samples. MiR-378 regulates systemic energy homeostasis and the oxidative capacity of insulin target tissues by repressing carnitine *O*-acetyltransferase and *MED13* [51]. Expression of miR-378a increased in adipose tissue of high fat diet-induced obese mice and during differentiation of preadipocytes [52]. Decreased expression of *MAPK1*, a target of miR-378a, lead to an induction of adipogenesis [52].

The miR-7 family is an important regulator in fetal pancreas development. In humans, it is the most abundantly expressed miRNA in the pancreatic islet [20]. Nieto et al found that inhibition of miR-7 during early embryogenesis resulted in underdeveloped pancreatic tissue, reduced insulin, and increased glucose intolerance[53]. Additionally inhibition of miR-7 led to increased beta cell death and decreased insulin production[53]. MiR-7 also regulates insulin granule exocytosis from mature pancreatic beta-cells [54, 55].

Several studies describe increased circulating concentrations of miR-885-5p and liver pathologies including fatty liver disease and hepatocellular carcinoma[56] but not much is known about its role in fetal development. MiR-885 is a biomarker for pancreatic cancer, hepatocellular carcinoma, cirrhosis and chronic hepatitis B [57–60]. One of the potential targets of miR-885-5p is *ABCA1*, a key gene involved in reverse cholesterol transport and phospholipid homeostasis in both liver and placenta [37]. In female PHFH exposed to maternal obesity, we found an enrichment of miR-885-5p expression but no change in *ABCA1* mRNA expression, but in male PHFH exposed to maternal obesity there was no change in miR-885-5p expression but a strong increase in *ABCA1* expression (Figure 2 and 3). The findings confirm the sex specific findings from miRNAs and target genes above and support the need for additional investigations to fully describe the mechanisms responsible for sex-specific metabolic programming effects.

Our findings are limited by the fact that we only assayed AF at one time period during second trimester of gestation. The GDM AF miRNA enrichment was validated in a separate cohort of second trimester PHFH and the consistent increases in miR-199a-3p, miR-503-5p and miR-885-5p in both cohorts adds confidence to our findings. Since amniocentesis is a rare medical procedure due to a recent switch to cell-free fetal DNA testing for cytogenetic studies, we were unable to validate our AF findings in a separate cohort of AF samples. However, we were able to confirm our findings in a cohort of PHFH obtained from women with obesity who had not yet been tested for GDM. Given the small sample size and the increased maternal age in the AF sample cohort, our findings may not be applicable to other populations of women and their offspring. Additional limitations that may have affected miRNA expression include the inability to study the effect of maternal BMI since these data were not collected and the sex specific trends in GDM offspring birth weight (Table 1). Due to study design and the inherent properties of miRNAs, we were unable to determine if the enriched miRNAs were derived from fetal tissues, placenta or maternal tissues. Additional mechanistic studies are needed to determine not only the origin of the differentially

expressed miRNAs but also how they travel to target tissue. Finally, we employed a candidate miRNA approach based on literature review, and may have missed important miRNAs critical to regulating fetal development in the intrauterine environment affected by GDM that may have been detected with an unbiased approach.

In summary, the intrauterine environment associated with GDM and maternal obesity is associated with an increase in miR-885-5p, miR-199a-3p, miR-1268a, miR-7-1-3p and miR-503-5p in second trimester AF and fetal liver exposed to maternal obesity has consistent enrichment of the same miRNAS with a corresponding decrease in expression of target genes *ABCA1*, PAK4 and *INSR*. These findings suggest that miRNAs may be a critical component to maternal-placental-fetal communication during gestation and therefore have the potential to affect fetal development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Jeanne Manson, PhD and Deborah Driscoll, MD for their work in establishing the amniotic fluid and amniocyte biospecimen repository.

Competing interests/Grants/Funding: The authors have no competing financial interests to declare. Research reported in this publication was supported by the National Institute of Environmental Health Sciences and the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award numbers SEP: K08 DK090302, P30 ES013508, UL1TR001878, the McCabe Foundation and the creation of the biospecimen repository: 5R21-ES11675. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

1. Ashino NG, Saito KN, Souza FD, Nakutz FS, Roman EA, Velloso LA et al. Maternal high-fat feeding through pregnancy and lactation predisposes mouse offspring to molecular insulin resistance and fatty liver. J Nutr Biochem 2012; 23(4): 341–8. [PubMed: 21543214]
2. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 2005; 115(3): e290–6. [PubMed: 15741354]
3. Catalano PM, Ehrenberg HM. The short- and long-term implications of maternal obesity on the mother and her offspring. Bjog 2006; 113(10): 1126–33. [PubMed: 16827826]
4. Crume TL, Ogden L, West NA, Vehik KS, Scherzinger A, Daniels S et al. Association of exposure to diabetes in utero with adiposity and fat distribution in a multiethnic population of youth: the Exploring Perinatal Outcomes among Children (EPOCH) Study. Diabetologia 2011; 54(1): 87–92. [PubMed: 20953862]
5. Kawasaki M, Arata N, Ogawa Y. Obesity and abnormal glucose tolerance in the offspring of mothers with diabetes. Curr Opin Obstet Gynecol 2018.
6. McCurdy CE, Bishop JM, Williams SM, Grayson BE, Smith MS, Friedman JE et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. The Journal of clinical investigation 2009; 119(2): 323–35. [PubMed: 19147984]
7. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia 1993; 36(1): 62–7. [PubMed: 8436255]
8. Plagemann A Perinatal programming and functional teratogenesis: impact on body weight regulation and obesity. Physiol Behav 2005; 86(5): 661–8. [PubMed: 16280141]

9. Park JH, Stoffers DA, Nicholls RD, Simmons RA. Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. J Clin Invest 2008; 118(6): 2316–24. [PubMed: 18464933]
10. Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W, Schoenleben M, Markert UR. Pregnancy-associated miRNA-clusters. J Reprod Immunol 2013; 97(1): 51–61. [PubMed: 23432872]
11. Mouillet JF, Chu T, Sadovsky Y. Expression patterns of placental microRNAs. Birth Defects Res A Clin Mol Teratol 2011; 91(8): 737–43. [PubMed: 21425434]
12. Mouillet JF, Ouyang Y, Coyne CB, Sadovsky Y. MicroRNAs in placental health and disease. Am J Obstet Gynecol 2015; 213(4 Suppl): S163–72. [PubMed: 26428496]
13. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ et al. The microRNA spectrum in 12 body fluids. Clin Chem 2010; 56(11): 1733–41. [PubMed: 20847327]
14. Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. J Perinatol 2005; 25(5): 341–8. [PubMed: 15861199]
15. Pinney SE, Mesaros CA, Snyder NW, Busch CM, Xiao R, Aijaz S et al. Second trimester amniotic fluid bisphenol A concentration is associated with decreased birth weight in term infants. Reprod Toxicol 2017; 67: 1–9. [PubMed: 27829162]
16. O'Neill K, Alexander J, Azuma R, Xiao R, Snyder NW, Mesaros CA et al. Gestational Diabetes Alters the Metabolomic Profile in 2nd Trimester Amniotic Fluid in a Sex-Specific Manner. Int J Mol Sci 2018; 19(9).
17. Lazaro CA, Croager EJ, Mitchell C, Campbell JS, Yu C, Foraker J et al. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology 2003; 38(5): 1095–106. [PubMed: 14578848]
18. Alejandro EU, Gregg B, Wallen T, Kumusoglu D, Meister D, Chen A et al. Maternal diet-induced microRNAs and mTOR underlie beta cell dysfunction in offspring. J Clin Invest 2014; 124(10): 4395–410. [PubMed: 25180600]
19. Collares CV, Evangelista AF, Xavier DJ, Rassi DM, Arns T, Foss-Freitas MC et al. Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. BMC Res Notes 2013; 6: 491. [PubMed: 24279768]
20. Correa-Medina M, Bravo-Egana V, Rosero S, Ricordi C, Edlund H, Diez J et al. MicroRNA miR-7 is preferentially expressed in endocrine cells of the developing and adult human pancreas. Gene Expr Patterns 2009; 9(4): 193–9. [PubMed: 19135553]
21. Guay C, Regazzi R. Circulating microRNAs as novel biomarkers for diabetes mellitus. Nat Rev Endocrinol 2013; 9(9): 513–21. [PubMed: 23629540]
22. Hu L, Han J, Zheng F, Ma H, Chen J, Jiang Y et al. Early second-trimester serum microRNAs as potential biomarker for nondiabetic macrosomia. Biomed Res Int 2014; 2014: 394125. [PubMed: 25405200]
23. Lazzarini R, Olivieri F, Ferretti C, Mattioli-Belmonte M, Di Primio R, Orciani M. mRNAs and miRNAs profiling of mesenchymal stem cells derived from amniotic fluid and skin: the double face of the coin. Cell Tissue Res 2014; 355(1): 121–30. [PubMed: 24104559]
24. Liu T, Chen Q, Huang Y, Huang Q, Jiang L, Guo L. Low microRNA-199a expression in human amniotic epithelial cell feeder layers maintains human-induced pluripotent stem cell pluripotency via increased leukemia inhibitory factor expression. Acta Biochim Biophys Sin (Shanghai) 2012; 44(3): 197–206. [PubMed: 22285730]
25. Miranda-Sayago JM, Fernandez-Arcas N, Reyes-Engel A, Benito C, Narbona I, Alonso A. Changes in CDKN2D, TP53, and miR125a expression: potential role in the evaluation of human amniotic fluid-derived mesenchymal stromal cell fitness. Genes Cells 2012; 17(8): 673–87. [PubMed: 22747700]
26. Pillar N, Yoffe L, Hod M, Shomron N. The possible involvement of microRNAs in preeclampsia and gestational diabetes mellitus. Best Pract Res Clin Obstet Gynaecol 2015; 29(2): 176–82. [PubMed: 25214435]
27. Rayner KJ, Hennessy EJ. Extracellular communication via microRNA: lipid particles have a new message. J Lipid Res 2013; 54(5): 1174–81. [PubMed: 23505318]
28. Ross SA, Davis CD. The emerging role of microRNAs and nutrition in modulating health and disease. Annu Rev Nutr 2014; 34: 305–36. [PubMed: 25033062]

29. Tryggestad JB, Vishwanath A, Jiang S, Mallappa A, Teague AM, Takahashi Y et al. Influence of gestational diabetes mellitus on human umbilical vein endothelial cell miRNA. Clin Sci (Lond) 2016; 130(21): 1955–67. [PubMed: 27562513]
30. Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC et al. MicroRNA-223 coordinates cholesterol homeostasis. Proc Natl Acad Sci U S A 2014; 111(40): 14518–23. [PubMed: 25246565]
31. Zhao C, Dong J, Jiang T, Shi Z, Yu B, Zhu Y et al. Early second-trimester serum miRNA profiling predicts gestational diabetes mellitus. PLoS One 2011; 6(8): e23925. [PubMed: 21887347]
32. Zhao C, Zhang T, Shi Z, Ding H, Ling X. MicroRNA-518d regulates PPARalpha protein expression in the placentas of females with gestational diabetes mellitus. Mol Med Rep 2014; 9(6): 2085–90. [PubMed: 24639097]
33. Zhu Y, Tian F, Li H, Zhou Y, Lu J, Ge Q. Profiling maternal plasma microRNA expression in early pregnancy to predict gestational diabetes mellitus. Int J Gynaecol Obstet 2015; 130(1): 49–53. [PubMed: 25887942]
34. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3(7): RESEARCH0034.
35. Qi R, Weiland M, Gao XH, Zhou L, Mi QS. Identification of endogenous normalizers for serum microRNAs by microarray profiling: U6 small nuclear RNA is not a reliable normalizer. Hepatology 2012; 55(5): 1640–2; author reply 1642–3.
36. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife 2015; 4.
37. Stefulj J, Panzenboeck U, Becker T, Hirschmugl B, Schweinzer C, Lang I et al. Human endothelial cells of the placental barrier efficiently deliver cholesterol to the fetal circulation via ABCA1 and ABCG1. Circ Res 2009; 104(5): 600–8. [PubMed: 19168441]
38. Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q et al. Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. Cancer Cell 2011; 19(2): 232–43. [PubMed: 21316602]
39. Lingwood BE, Henry AM, d'Emden MC, Fullerton AM, Mortimer RH, Colditz PB et al. Determinants of body fat in infants of women with gestational diabetes mellitus differ with fetal sex. Diabetes Care 2011; 34(12): 2581–5. [PubMed: 21994428]
40. Regnault N, Gillman MW, Rifas-Shiman SL, Eggleston E, Oken E. Sex-specific associations of gestational glucose tolerance with childhood body composition. Diabetes Care 2013; 36(10): 3045–53. [PubMed: 23877978]
41. Retnakaran R, Kramer CK, Ye C, Kew S, Hanley AJ, Connelly PW et al. Fetal sex and maternal risk of gestational diabetes mellitus: the impact of having a boy. Diabetes Care 2015; 38(5): 844–51. [PubMed: 25693837]
42. Alexander J, Teague AM, Chen J, Aston CE, Leung YK, Chernausek S et al. Offspring sex impacts DNA methylation and gene expression in placentae from women with diabetes during pregnancy. PLoS One 2018; 13(2): e0190698. [PubMed: 29470513]
43. He L, Tang M, Xiao T, Liu H, Liu W, Li G et al. Obesity-Associated miR-199a/214 Cluster Inhibits Adipose Browning via PRDM16-PGC-1alpha Transcriptional Network. Diabetes 2018; 67(12): 2585–2600. [PubMed: 30279164]
44. Dimasuay KG, Boeuf P, Powell TL, Jansson T. Placental Responses to Changes in the Maternal Environment Determine Fetal Growth. Front Physiol 2016; 7: 12. [PubMed: 26858656]
45. Jansson T, Aye IL, Goberdhan DC. The emerging role of mTORC1 signaling in placental nutrient-sensing. Placenta 2012; 33 Suppl 2: e23–9. [PubMed: 22687819]
46. Szabo G, Bala S. MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol 2013; 10(9): 542–52. [PubMed: 23689081]
47. Caporali A, Meloni M, Vollenkle C, Bonci D, Sala-Newby GB, Addis R et al. Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. Circulation 2011; 123(3): 282–91. [PubMed: 21220732]

48. Shantikumar S, Caporali A, Emanueli C. Role of microRNAs in diabetes and its cardiovascular complications. Cardiovasc Res 2012; 93(4): 583–93. [PubMed: 22065734]

49. Wang XH, Qian RZ, Zhang W, Chen SF, Jin HM, Hu RM. MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. Clin Exp Pharmacol Physiol 2009; 36(2): 181–8. [PubMed: 18986336]

50. Zhou B, Ma R, Si W, Li S, Xu Y, Tu X et al. MicroRNA-503 targets FGF2 and VEGFA and inhibits tumor angiogenesis and growth. Cancer Lett 2013; 333(2): 159–69. [PubMed: 23352645]

51. Carrer M, Liu N, Grueter CE, Williams AH, Frisard MI, Hulver MW et al. Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378*. Proc Natl Acad Sci U S A 2012; 109(38): 15330–5. [PubMed: 22949648]

52. Huang N, Wang J, Xie W, Lyu Q, Wu J, He J et al. MiR-378a-3p enhances adipogenesis by targeting mitogen-activated protein kinase 1. Biochem Biophys Res Commun 2015; 457(1): 37–42. [PubMed: 25529446]

53. Nieto M, Hevia P, Garcia E, Klein D, Alvarez-Cubela S, Bravo-Egana V et al. Antisense miR-7 impairs insulin expression in developing pancreas and in cultured pancreatic buds. Cell Transplant 2012; 21(8): 1761–74. [PubMed: 22186137]

54. Filios SR, Shalev A. beta-Cell MicroRNAs: Small but Powerful. Diabetes 2015; 64(11): 3631–44. [PubMed: 26494215]

55. Kredo-Russo S, Mandelbaum AD, Ness A, Alon I, Lennox KA, Behlke MA et al. Pancreas-enriched miRNA refines endocrine cell differentiation. Development 2012; 139(16): 3021–31. [PubMed: 22764048]

56. Raitoharju E, Seppala I, Lyytikainen LP, Viikari J, Ala-Korpela M, Soininen P et al. Blood hsa-miR-122–5p and hsa-miR-885-5p levels associate with fatty liver and related lipoprotein metabolism-The Young Finns Study. Sci Rep 2016; 6: 38262. [PubMed: 27917915]

57. Hur K, Toiyama Y, Schetter AJ, Okugawa Y, Harris CC, Boland CR et al. Identification of a metastasis-specific MicroRNA signature in human colorectal cancer. J Natl Cancer Inst 2015; 107(3).

58. Mitsuhashi K, Yamamoto I, Kurihara H, Kanno S, Ito M, Igarashi H et al. Analysis of the molecular features of rectal carcinoid tumors to identify new biomarkers that predict biological malignancy. Oncotarget 2015; 6(26): 22114–25. [PubMed: 26090613]

59. Schultz NA, Dehlendorff C, Jensen BV, Bjerregaard JK, Nielsen KR, Bojesen SE et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. JAMA 2014; 311(4): 392–404. [PubMed: 24449318]

60. Tan Y, Ge G, Pan T, Wen D, Gan J. Serum MiRNA panel as potential biomarkers for chronic hepatitis B with persistently normal alanine aminotransferase. Clin Chim Acta 2015; 451(Pt B): 232–9. [PubMed: 26483130]

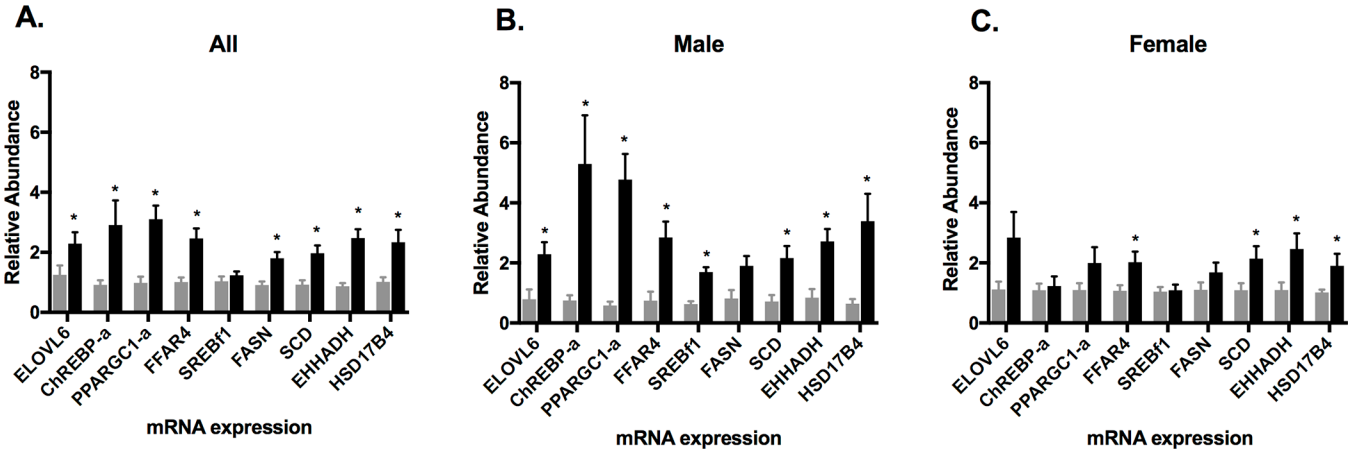

Figure 1:

mRNA expression for genes involved in *de novo* lipogenesis in primary human fetal hepatocytes (PHFH) exposed to maternal obesity. * $p<0.05$. Data shown as mean ± SEM. A. All samples (n=7–10). B. Male samples (n=4–7). C. Female samples (n=−35). Gray bars = maternal BMI < 25 (control); Black bars = maternal BMI > 30 (obese). mRNA expression normalized to β-actin.

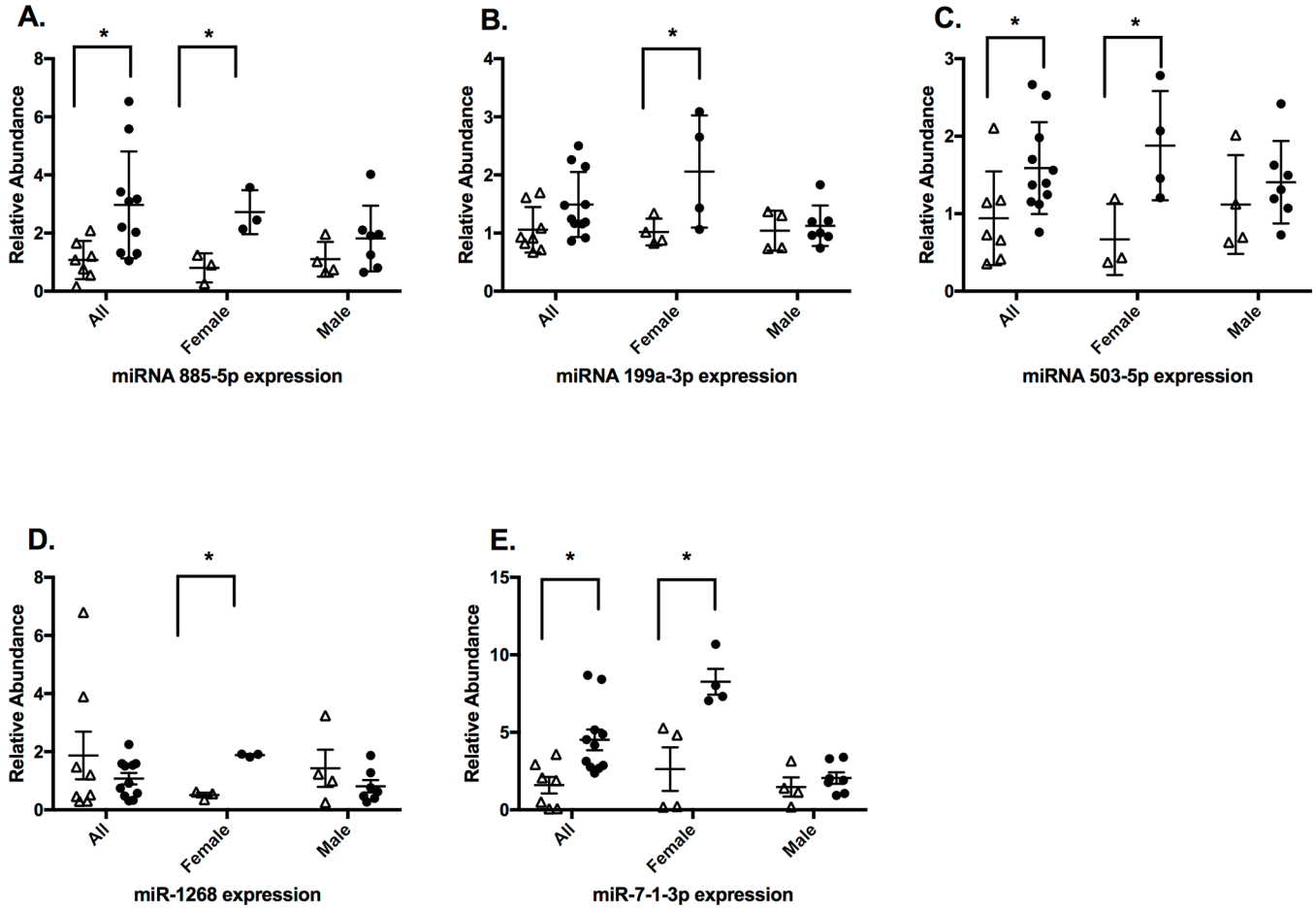

Figure 2:

miRNA expression in obese exposed PHFH A. miR-885-5p; B.miR-199-30; C: miR-503-5p; D. miR-1268a; E. miR-7-1-3p. Control: open triangles; Obesity exposed: closed circles. N=7–10 (all); n=3–5 (female) and n=4–7 (male). Exact number of samples per experiment represented on figure.* $p<0.05$. Data shown as mean ± SEM. A. MiRs normalized to miR-16.

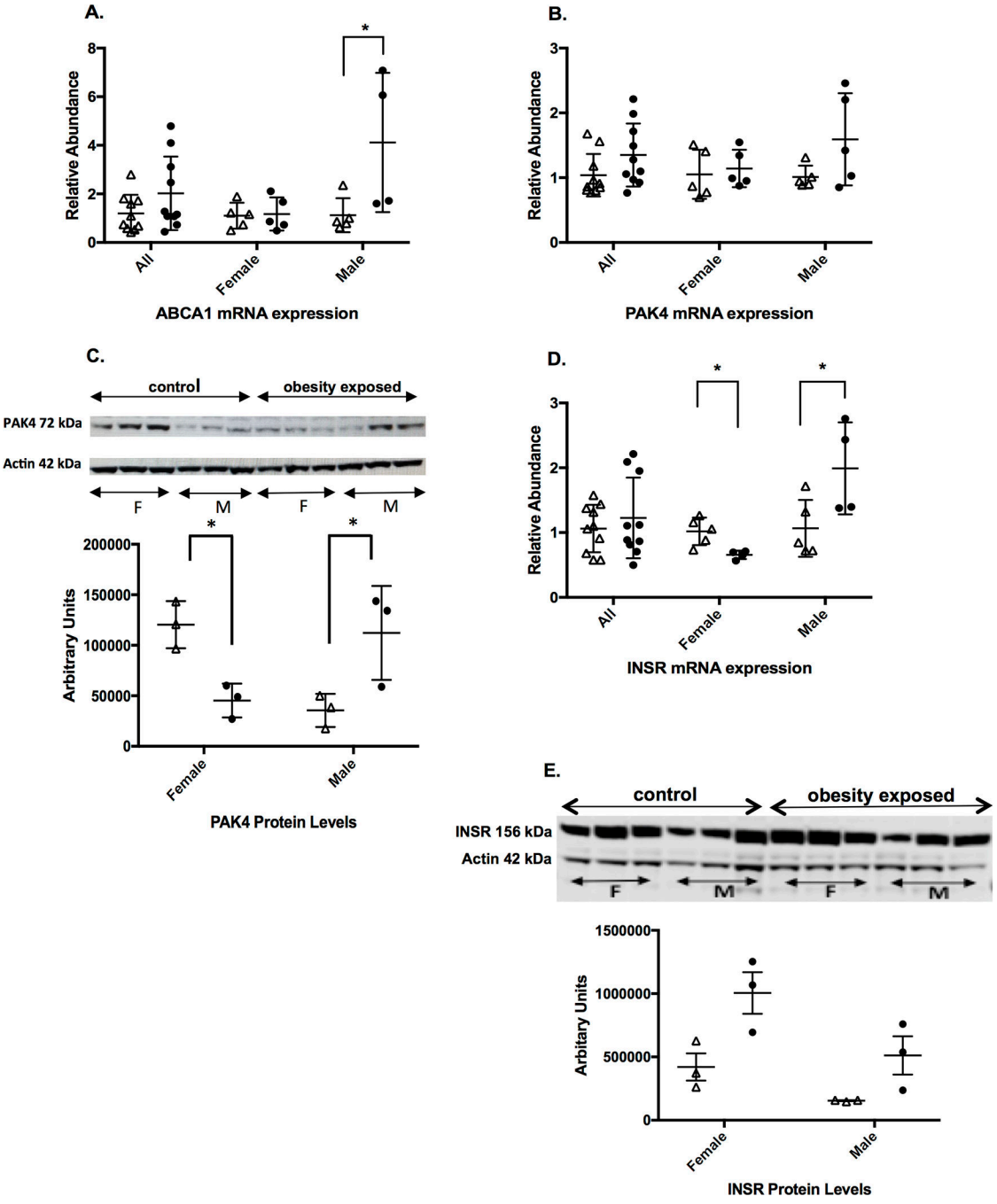

Figure 3:

mRNA and protein expression in obese exposed PHFH. A. *ABCA1* mRNA expression normalized. B. *PAK4* mRNA expression. C. PAK4 protein levels. D. INSR mRNA expression. E. INSR protein levels. Control: open triangles; Obesity exposed: closed circles. N=7–10 (all); n=3–5 (female) and n=4–7 (male). Exact number of samples per experiment represented on figure.* $p<0.05$. Data shown as mean ± SEM. mRNA and protein levels normalized to β-actin.

Table 1.

Maternal and infant demographics for AF samples. AF: amniotic fluid, GDM: gestational diabetes, GA: gestational age, NS: non-significant.

Characteristic	GDM	Control	P value
Race, N (%)			
White	18 (90)	18 (90)	NS
Asian/Pacific Islander	2 (10)	2 (10)	NS
Ethnicity, N (%)			
Non-Hispanic	19 (95)	20 (100)	NS
Hispanic	1 (5)	0 (0)	NS
Maternal age			
years, mean ± SD	37.4 ± 3.6	37.3 ± 2.8	0.73
GA at amniocentesis	16.2 ± 0.6	16.2 ± 0.5	1
weeks, mean ± SD	16.2 ± 0.6	16.2 ± 0.5	1
GA at birth			
weeks, mean ± SD	39.1 ± 1.4	38.9 ± 1.6	0.67
Birth weight			
grams, mean ± SD			
All offspring	3388.9 ± 525.8	3419.0 ± 479.9	0.83
Female offspring	3078.9 ± 411.4	3345.2 ± 553.2	0.16
Male offspring	3733.4 ± 422.0	3492.8 ± 409.8	0.08

Table 2.

Differentially expressed miRNAs in GDM exposed Amniotic Fluid

Sample group	miRNA	Fold change	P value	3P-seq tag
All (n=20 pairs)	hsa-mir-138–5p	0.70	0.03	GUGGUCG
	hsa-mir-199a-3p*	1.77	0.01	UGAUGAC
	hsa-mir-15b-5p	1.20	0.03	ACGACGA
	hsa-mir-503–5p*	1.50	0.03	GCGACGA
	hsa-mir-1268a*	1.74	0.03	GUGCGGG
Female (n=10 pairs)	hsa-mir-7–1-3p*	1.62	0.004	AAACAA
	hsa-mir-99a-5p	0.88	0.04	AUGCCCA
	hsa-mir-126–3p1	1.32	0.04	GCCAUGC
	hsa-mir-185–5p	1.35	0.001	AGAGAGG
	hsa-mir-210–3p	0.76	0.007	UGCGUGU
	hsa-mir-486–3p	1.41	0.01	ACGGGG
	hsa-mir-15b-5p	1.31	0.01	ACGACGA
	hsa-mir-378a-3p*	1.50	0.01	UCAGGUC
	hsa-mir-197–3p	1.19	0.02	CACCACU
	hsa-mir-885–5p*	1.64	0.02	CAUUACC
	hsa-mir-146a-5p	1.47	0.03	UCAAGAG
	hsa-mir-302a-3p	1.48	0.03	UCGUGAA
Male (n=10 pairs)	hsa-mir-199a-3p*	1.77	0.01	UGAUGAC

* FC ≥ 1.5 or ≤ 0.66 and $p<0.05$

Table 3:

Maternal and fetal demographic data for primary human fetal hepatocyte samples

Characteristic	Maternal Obesity	Control	P Value
Fetal Sex, N(%)	12	10	NS
Male	7 (58)	5 (50)	NS
Female	5 (42)	5 (50)	NS
Maternal BMI			
mean ± SD	35.1 ± 2.9	21.6 ± 1.7	<0.001
Maternal Age			
mean ± SD	24.3 ± 0.8	23.6 ± 0.7	NS
Gestational Age			
weeks, mean ± SD	18.7 ± 0.7	18.7 ± 0.8	NS

Maternal obesity exposed: maternal BMI >30; Control: maternal BMI < 25