2020

Population variations in placental micro-RNA expression by self identified maternal race

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

POPULATION VARIATIONS IN PLACENTAL MICRO-RNA EXPRESSION BY SELF IDENTIFIED MATERNAL RACE

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B.S., University of Wisconsin-Madison, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

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POPULATION VARIATIONS IN PLACENTAL MICRO-RNA EXPRESSION BY SELF IDENTIFIED MATERNAL RACE

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ABSTRACT

With differences in fetal growth patterns observed across racial demographics, interest in possible biological causes for these differences has increased. Prior works have studied the effects of insulin resistance in pregnancy on fetal fat deposition and growth *in utero*. One proposed mechanism to explain the physiological decrease in insulin sensitivity observed in normal pregnancy is the release of epigenetic factors such as placental miRNAs that have downstream effects on nutrient availability and fetal growth. The aim of this study was to identify placental miRNAs in women of different races and to establish expression patterns between these groups, specifically if expression patterns were related to measures of fetal growth and insulin resistance. Untargeted RNAseq and targeted RT-qPCR techniques were utilized for miRNA expression analysis and validation, respectively. Statistical modeling was used to interpret relationships between miRNA expression and maternal and neonatal body composition variables. qPCR results validated RNA sequencing data of differential miRNA expression between maternal racial groups. While miRNA-34c-5p and 192-5p fold changes were not correlated with maternal insulin resistance (as measured by HOMA-IR) in both non-Hispanic black and non-Hispanic white women, both miRNA-34c-5p and miRNA-192-5p were both positively correlated with neonatal body composition measures in neonates born to NHB women only. Further analysis of more miRNAs from additional RNAseq is planned.

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LIST OF ABBREVIATIONS

ΔΔCt.....Delta-Delta threshold Cycle

% FM .....Percent Fetal Fat Mass

AKT .....Protein kinase B

BMI.....Body Mass Index

FM.....Fat Mass (skinfolds)

HOMA-IR..... Homeostatic Model Assessment of Insulin Resistance

hPL.....human Placental Lactogen

IR.....Insulin Resistance

IRS .....Insulin Receptor substrate

LBM.....Lean Body Mass (skinfolds)

miRNA.....Micro RNA

mRNA.....messenger RNA

NHB .....Non-Hispanic black

NHW.....Non-Hispanic white

PI3-Kinase.....Phosphoinositide 3-kinase

ppBMI.....Pre-Pregnancy BMI

qPCR.....quantitative polymerase chain reaction

RIN.....RNA Integrity Number

RISC.....RNA-induced silencing complex

RNAseq.....RNA Sequencing

INTRODUCTION

The percentage of children and adolescents who are obese in the United States has more than tripled since the 1970's [1], with greater than 50% of the American population projected to be obese by 2030[2]. Birth weight, and in particular fetal adiposity, is a key marker of newborn health. There is increasing evidence that low birth weight and ponderal index (a standardized measure that accounts for both birth weight and height) are associated with later adverse health outcomes like increased risk of cardiovascular disease and stroke[3]. Macrosomic neonates (> 4000 - 4500grams in weight)[4], on the other hand, are at higher risk of developing obesity and insulin resistance in late childhood and adulthood [5,6].

Maternal obesity has been linked to adverse pregnancy outcomes and can have detrimental long-term effects beyond fetal development [7,8]. Obesity is a crucial indicator of maternal insulin resistance, noted by the inverse relationship between insulin sensitivity and maternal fat mass during early pregnancy [9,10]. Progressive maternal insulin resistance is associated with the degree of glucose flux to the fetus[11]; which is necessary for neonatal adipose tissue deposition[12]. Recent studies have suggested that maternal adiposity is primarily associated with increased neonatal adiposity in female offspring only[13,14] while male neonates have increased insulin sensitivity and secretion[15]. Maternal pre-gravid body mass index (**BMI**) has also been shown to be a strong predictor of childhood obesity and metabolic dysregulation in 8-year-old children [16]. There is a

significant interest in further understanding maternal metabolism changes in pregnancy as possible intervention points for improving and preventing obesity in future generations.

Insulin Synthesis and Secretion

A brief review of insulin's physiological role is necessary before discussing its ramifications in pregnancy and in neonatal outcomes. Insulin is an anabolic peptide hormone that is a critical component in regulating human metabolism [17,18]. Secreted by pancreatic β-cells in the islets of Langerhans, insulin functions to maintain glucose homeostasis by promoting glucose uptake for use in cellular respiration and metabolism[18]. After a series of posttranscriptional modifications involving cleavage by endonucleases [19] (Figure 1), the mature hormone is stored in secretory granules in the islets of Langerhans for secretion upon stimulation [20].

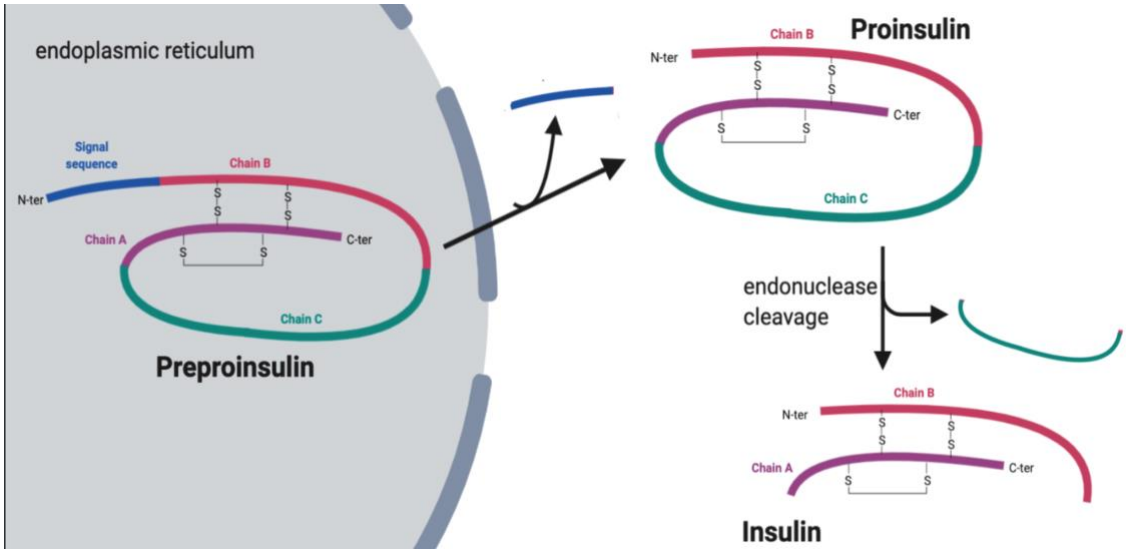

Figure 1. Post-translational modifications of insulin synthesis. Pre-proinsulin is cleaved of its signal sequence upon exit from the endoplasmic reticulum, forming proinsulin which is further cleaved by pancreatic peptidases into the mature form of insulin. Adapted from Colorado State Pathophysiology Online Module, (2020)[20].

Once stimulated, insulin undergoes exocytosis from the β-cell for active glucose homeostasis regulation. Following a meal, the amount of circulating glucose in the bloodstream increases which leads to the facilitated diffusion of glucose through the insulin-independent GLUT2 membrane transporter. Figure 2 demonstrates the signal cascade of intracellular glucose metabolism leading to ATP production, followed by the eventual depolarization of the cell by inhibition of ATP sensitive K-efflux channels. This depolarization leads to an influx of Ca+ ions via the opening of L-type voltage gated Ca+ channels, which stimulates the exostosis of insulin from their storage granules in the β-cell [21].

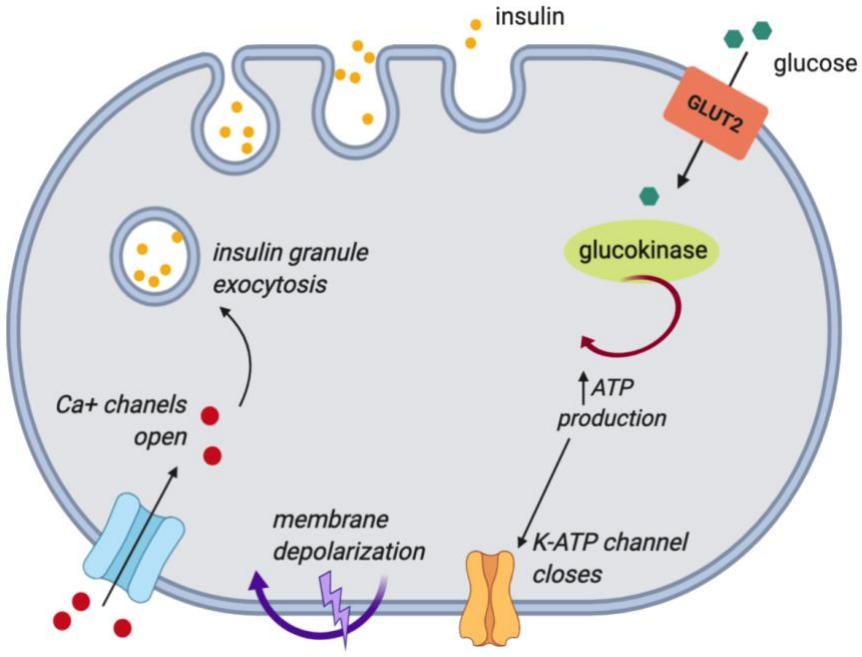

Figure 2. Insulin release for glucose homeostasis. Glucose entry into the β-cell through the GLUT2 transporter leads to a cascade of events including cellular depolarization and Ca+ ion influx to the cell, eventually causing insulin exocytosis. Adapted from Physiology—6th Edition (2017)[18]; Cho et al., (2018)[17]; and Weiss et al., (2000)[21].

Now in the bloodstream, insulin can bind to its transmembrane insulin receptor on target tissue cells and mediate glucose uptake as shown in Figure 3. The binding of insulin to its receptor's α subunit induces a conformational change which causes the autophosphorylation of several tyrosine residues present in the receptor's β subunit. These residues are recognized by the phosphotyrosine-binding domains of adaptor proteins such as the insulin receptor substrate family (**IRS**) protein, which becomes phosphorylated. This phosphorylation is then recognized by the Src Homology 2 domain of the p85 regulatory subunit of Phosphoinositide 3-kinase (**PI3-kinase**), effectively activating the PI3-kinase. The phosphorylation by the p110 catalytic subunit phosphorylates phosphatidylinositol (4,5) bisphosphate leads to the activation of Protein kinase B (also referred to as **AKT**). Both AKT and PI3-Kinase play critical roles in glucose uptake by inducing the translocation of the insulin dependent glucose transporter, GLUT4, from intracellular storage to the plasma membrane [17,21,22]. Once at the membrane surface, glucose is taken up by target cells via the ATP-dependent GLUT4 (like in skeletal muscle) for glycolysis and other metabolic functions (Figure 3)[23].

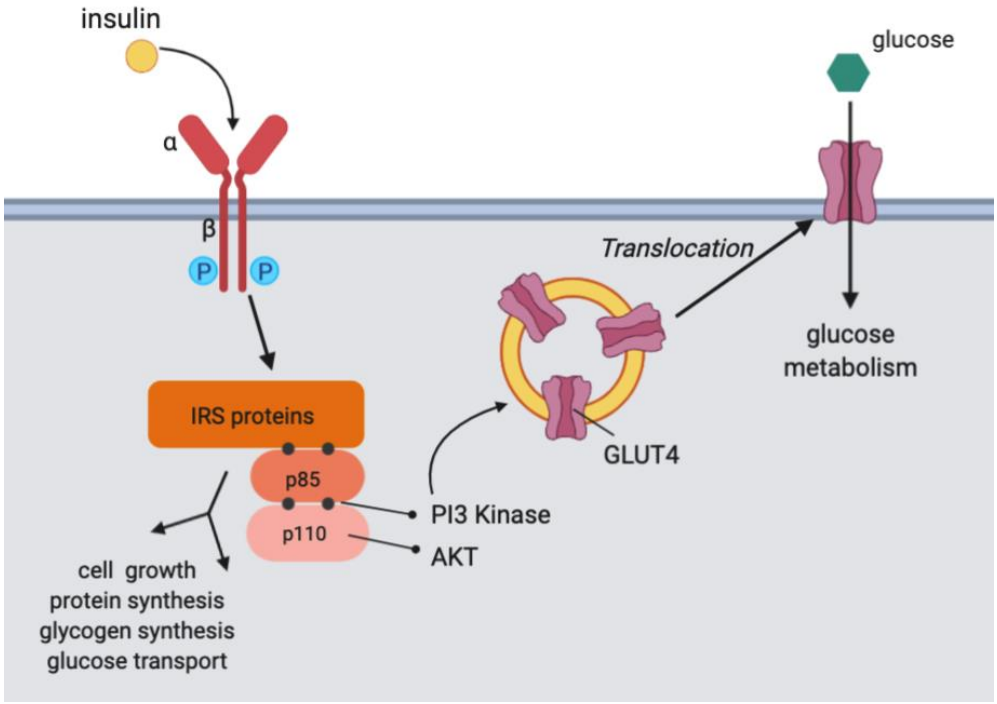

Figure 3. Insulin signaling of glucose uptake for homeostasis. Insulin binding to its receptor leads to a signal cascade of conformational changes and activation steps to result in the eventual translocation of GLUT4 to the cell surface. Adapted from Harrison's principles of internal medicine, 17th edition (2008)[23].

Insulin Signaling Adaptations in Pregnancy

In a nonpregnant healthy female, insulin release leads to a decrease in circulating glucose levels by several mechanisms, one being increased glucose uptake into target tissues [18]. During a healthy uncomplicated pregnancy, maternal metabolic adaptations occur in preparation for the increased energy demand of a growing fetus. These changes include diminished insulin sensitivity [24], altered glucose transport and cellular uptake [25], and increased androgen secretion leading in alterations in insulin binding frequencies [23]. Studies have shown as high as a 23% reduction in IRS-1 protein expression in mothers at the time of delivery compared to weight matched nonpregnant women [26].

Insulin resistance (**IR**), defined as the decreased ability of target tissues to respond to normal circulating concentrations of insulin [24], progresses throughout pregnancy plateauing in the late third trimester [27]. In uncomplicated obese pregnancies, insulin-stimulated glucose transport was decreased 32% in maternal skeletal muscle fibers when compared to a nonpregnant obese control group[28]. Maternal IR is a physiological adaptation to pregnancy, and is associated with higher maternal circulating free fatty acids and triglycerides[29] which are important for normal growth and fat deposition [16]. However greater than normal IR has been associated with poor maternal and fetal outcomes, including early onset type 2 diabetes mellitus in the mother after pregnancy [30] as well obesity and long term metabolic dysfunction in the offspring [16,31]. Interestingly, insulin sensitivity improves to near non-pregnant levels within 2-3 days of delivery [32]. In one study, enhanced insulin sensitivity postpartum was accompanied by more than a 40% increase in insulin receptor concentration when compared to skeletal muscle tissue biopsies during late pregnancy[26]. Since the placenta is delivered following the fetus, it is suspected to play a critical role in this dramatic decrease in insulin resistance.

The Placental Interface

The placenta is a highly specialized hemochorial villous organ that is crucial in maintenance and progression of a pregnancy. It serves as the interface between maternal and fetal circulatory systems by way of direct contact between maternal blood and placental trophoblast cells [33,34]. The formation of the utero-placental unit depends on the coordinated interaction of both fetal derived tissue from the chorionic sac (also known as

the chorionic plate in a mature placenta) and maternal tissue derived from the endometrium (referred to as the basal plate of the placenta) [35]. The intervillous space separates these two regions, and has extensively packed villous structures lined by a multinucleated syncytiotrophoblast [36]. At the ends of these chorionic villi is where a majority of the maternal-fetal exchange occurs [35,37], with maternal blood entering via the spiral endometrial arteries, pooling in the intervillous space and to the fetus by way of the umbilical vein [33,38] (Figure 4).

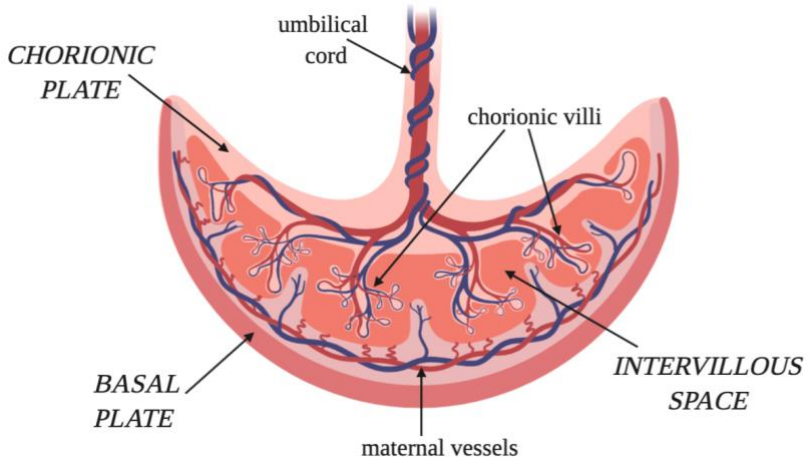

Figure 4. Maternal-fetal interface. Nutrient, gas and waste exchange occurs between fetus and mother by way of intervillous space. Adapted from SIUC School of Medicine Online Lecture (2003)[39].

Connected to the umbilical cord, the placenta serves respiratory, excretory, nutritional, immunologic and endocrine roles to support the growing fetus in addition to maintaining the pregnancy and preparing the mother for eventual labor [40,41]. The intimate relationship between fetus and mother via the placenta has posed numerous questions of possible nutritional and physiological imbalances that can affect both mother and fetus.

Placental metabolic reprogramming has been suggested to be an initial or early step in the progression and worsening of fetal growth restriction throughout pregnancy [42]. While the placenta is known to make substances that play important roles in driving maternal physiological adaptations during pregnancy [43], no such substance has yet been found to account for changes in insulin sensitivity. Secreted from the placenta, hormone placental lactogen (**hPL**) has been believed to promote the partial redirection of energy substrates to the growing fetus [44]. hPL has also been implicated in regulating the increase in maternal islet cell mass throughout a benign pregnancy [45]. Plasma tumor necrosis factor alpha (commonly referred to in literature as TNF-α) has been shown to cause insulin resistance through impairment of insulin signaling [9], but further research is necessary to understand the notable improvement of insulin sensitivity immediately following birth [32,46].

Placental miRNA Hypothesis for Insulin Resistance in Pregnancy

One recent proposed explanation for the shift in glucose transport between mother and fetus are placental-derived Micro RNAs [ribonucleic acids] (**miRNA**) [47]. As a class of noncoding RNAs, miRNAs play an important role in the downregulation of post-transcriptional gene expression and gene silencing via messenger RNA (**mRNA**) degradation and translational repression [48]. Through a sequence of processing steps as described in Figure 5, immature primary miRNA undergoes nuclear processing by Drosha (a ribonuclease protein) before it is exported into the cytoplasm[49]. The miRNA then undergoes secondary processing in the cytoplasm by cleavage from the Dicer enzyme, yielding a mature miRNA between 18-25 nucleotides in size [49,50]. This

biologically active miRNA is loaded onto an RNA-induced silencing complex (**RISC**), where it can then base pair to complementary mRNA sequences and regulate gene expression by means of RNA interference [51].

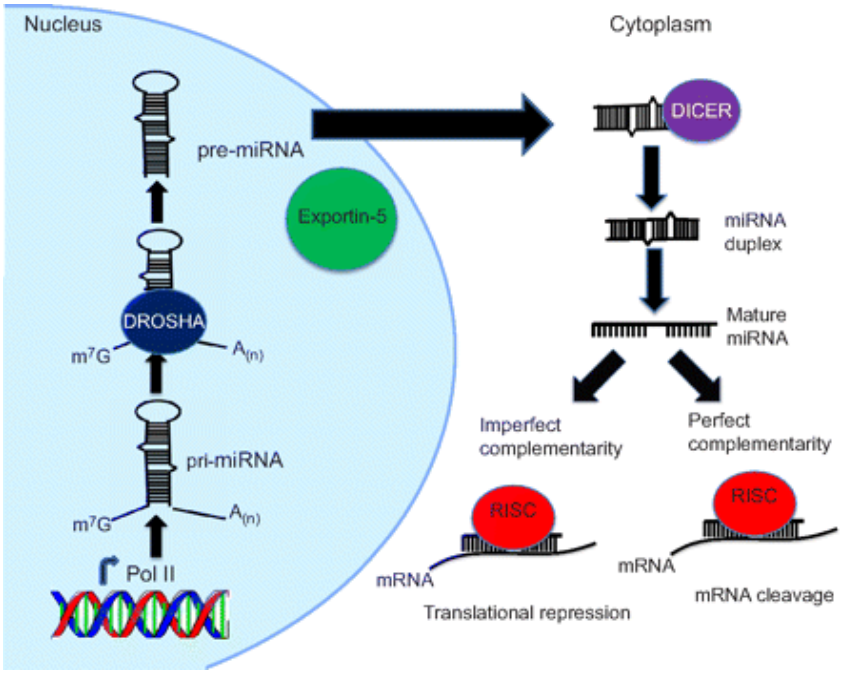

Figure 5. miRNA biogenesis and processing. Multi-step processing occurs before the noncoding molecule binds to a complementary mRNA and silences it, resulting in downstream decreased gene expression or gene silencing. Taken from Smith-Vikos and Slack (2012)[52].

Since their discovery in 1993, miRNAs have been found to play key roles in aging [52,53], cellular dysfunction and cancer [54]. In recent years, multiple miRNAs have been identified to play regulatory roles in pancreatic β-cell function during pregnancy [55]. Of particular interest, the C19MC miRNA cluster is found only in the primate species genome and is almost exclusively expressed in the placenta [56]. It [C19MC miRNA cluster] has been shown to be highly expressed in trophoblasts during pregnancy and subsequently eliminated from the maternal blood following delivery [56,57]. While the full

list of biological actions of the C19MC miRNA cluster remains to be identified, early work has shown that genes within this cluster are implicated in extravillous trophoblast migration [58], as well as cell proliferation and differentiation [56,59].

With minimal literature and knowledge of the full functions of placental miRNAs and the notable improvement of insulin sensitivity observed following placental delivery, there is interest in understanding if these miRNAs play a role in the maternal-placental-fetal crosstalk that contributes to nutrient availability and fetal growth by way of maternal insulin resistance (Figure 6). We propose that placental miRNAs cause a downstream signaling cascade that results in decreased insulin sensitivity and/or decrease in glucose uptake by the maternal skeletal muscle cells, which results in greater levels of circulating plasma glucose available for the growing fetus.

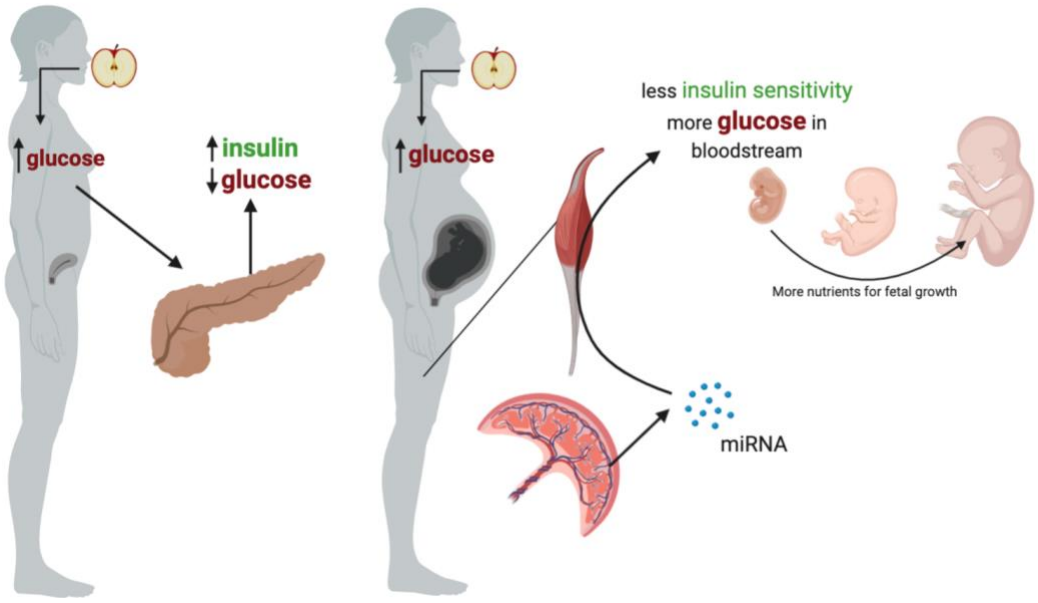

Figure 6. Insulin resistance by placental miRNA maternal-fetal crosstalk hypothesis. A proposed mechanism of placental miRNA signaling of maternal insulin sensitivity and corresponding glucose affects to the mother and fetus. Left: Nonpregnant

insulin signaling. Right: Insulin signaling adaptations in pregnancy with proposed miRNA mediator. Adapted from Catalano et. al (2009)[16].

Differences in Maternal-Fetal Outcomes by Race

With the many advancements made in studying and understanding maternal-fetal outcomes, particularly in the United States, there is limited understanding of how these physiological changes in pregnancy are influenced by race when compared to other fields such as cancer and heart disease. Infants of non-Hispanic black (**NHB**) mothers are twice as likely to be classified as low birth weight (<2,500 g) at the time of full-term delivery compared to neonates of non-Hispanic white (**NHW)** mothers [60,61]. Additionally, NHB obese women have a lower rate of macrosomia (birthweight > 4,000g regardless of gestational age[4]) compared to their obese NHW and Asian counterparts [62]. Overall, 22% of NHB women are considered obese while only 14% of NHW women are[63]. Even when adjusting for maternal factors such as age, parity, education status and initialization of care, NHB women are twice as likely to have a child of low birth weight compared to NHW women [64]. Also, the overall trend in decreasing birthweights in America is most prominent in infants born to NHB mothers than in those born to NHW women [65]. Similarly, term normal weight and size NHB neonates born following an uncomplicated pregnancy course have been reported to be smaller (by weight and length) than their NHW counterparts.

Research now indicates that these observed decreased birth weights among NHB neonates is attributable to less lean body mass as opposed to less adiposity as previously proposed [66]. The biological cause for this phenomenon is not yet fully understood. While

the CDC predicted in 2015 that just over half (50.2%) of infants born would be of racial or ethnic minorities (of non-NHW racial identity) [63], much of the current American literature on biological components of maternal-fetal health discusses NHW outcomes. A majority of the research previously mentioned on insulin resistance in pregnancy has also been conducted on cohorts of primarily NHW women, with limited understanding if these relationships are generalizable for minority women and their children. Furthermore, NHB women are less likely to drink [67] and smoke tobacco[68] throughout pregnancy than NHW mothers; both known causes of intrauterine growth restriction and overall smaller neonates [69,70], despite the above noted neonatal trends. There is limited information currently available if these relationships of insulin resistance, nutrient availability and neonate outcomes hold in non-Hispanic black women.

SPECIFIC AIMS

Studies have suggested that NHB women have smaller neonates [66], as well as higher instances of compounding social inequalities such as maternal age, obstetric follow up and overall access to medical care and education [1]. As NHB neonates show differential body composition [60,61,64] and pediatric growth patterns (specifically increased velocity of growth and weight gain) than NHW infants[71], insight into the causes of these differences is crucial to improve care practices and recommendations in both obstetrics and pediatrics. Furthermore, there is limited information currently available if the previously studied relationships of maternal insulin resistance and nutrient availability *in utero* with neonatal outcomes are applicable to minorities.

No studies have directly evaluated placental derived miRNAs as a potential mechanism for variations in maternal insulin resistance. This study aims to investigate the expression patterns of placental miRNAs in NHB and NHW women and identify associations with maternal insulin resistance and fetal body composition. We hypothesize that placental miRNA expression will be associated with similar patterns of insulin resistance, and their overall association will differ when comparing by maternal race. This study utilized samples from a placental bank of noncomplicated pregnancies to analyze the relationship between miRNA expression and insulin resistance by means of RNA sequencing and quantitative polymerase chain reaction (**qPCR**) validation. Accompanying statistical analysis was performed to elucidate the correlation and inter-relationship between these factors with respect to maternal race.

METHODS

Sample Collection

A cross sectional analysis was performed on a cohort of 508 healthy women recruited at term (>37 weeks' gestation) who delivered by scheduled elective cesarean at MetroHealth Medical Center in Cleveland OH. Exclusion criteria included multiples pregnancies, current illicit drug use, gestational or type 1 diabetes mellitus in current or prior pregnancies, hypertension before or during pregnancy, preeclampsia and other similar pregnancy related complications that pose metabolic and physiological alterations to mother and fetus. Uncomplicated obesity (as defined below) was not an exclusion criterion. Written and informed consent was obtained prior to participation and the study was approved by both the Tufts Institutional Review Board (IRB#12842) and the MetroHealth Institutional Review Board (IRB #13-00650). Placenta tissue was collected at the time of delivery from the maternal face of the placenta, halfway between the cord insertion and margins. Villous samples from across the placenta from different cotyledons (excluding basal plate and maternal decidua) were collected within 30 minutes of delivery. Tissue from multiple locations was combined for RNA extraction, as placental gene expression is heterogenous from cotyledon to cotyledon. Infant body composition was measured within 48 hours of birth using skinfold calipers [72].

PART 1. Initial RNAseq samples

Sample Selection

In accordance with an ongoing study, women with normal glucose tolerance were stratified by their pre-pregnancy BMI [(lean <25 kg/m^2 and (obese >30 kg/m^2)] and then

by neonatal adiposity. 17 NHB and 23 NHW maternal-placental-fetal triads were selected from the larger cohort. Nonsmoking mothers were selected when possible, due to the known associations with maternal smoking history and lower fetal birth weights [73]. RNA was extracted from these 40 samples and bioanalyzed as detailed below (methods part 2, pages 19-21). Previously generated RNAseq data for these 40 samples was validated by qPCR to establish differential patterns of placental miRNA expression between NHB and NHW women.

RNAseq

RNA sequencing (**RNAseq**) was performed by the Génome Québec Innovation Centre in accordance with their Illumina sequencing protocol for RNAseq. Sample aliquots were resuspended in commercial RNAse free water before they were sent for analysis. For small RNA profiling (< 200nt), all sample aliquots sent for sequencing were of 160 ng/uL minimal concentration, had RNA integrity numbers (**RIN**) >6.5, and contained 2400 ng of tissue suspended in 15uL volumes as recommended by the sequencing facility.

RNAseq analysis

Following RNA sequencing at the Génome Québec Innovation Centre, data analysis was performed by our biostatistics collaborator, Tianjiao Chu, at McGee-Women's Research Institute. Small RNA libraries were analyzed using an in-house-developed sequencing data analysis pipeline. Filtered reads were aligned against the human reference genome (GRCh38) using Bowtie technique, and the aligned sequences were then matched to annotated genes in GENCODE. The Bioconductor package

DESeq2 was used to identify placental miRNAs with significantly different level in placentas between NHB and NHW women.

RNAseq validation by targeted qPCR

Placental miRNA Primer selection

Statistically significant differences in placental miRNAs expressed between NHB and NHW women, with relative expression values greater than 6, were selected for validation and further study. These miRNAs of interest were then processed for potential gene targets of insulin signaling or glucose metabolism using TargetScan, a web server used to predict biological targets by matching sites of seed regions in placental miRNAs [74]. Next, functional analysis of the genes of interest was run through NCBI – Gene portal [75], where genes, associated miRNAs, and their known gene annotations can be compared to a database of confirmed functional gene analysis. The placental miRNAs with relatively high expression and those which had multiple notable genes of interest where chosen for qPCR analysis.

qPCR validation

Quantitative polymerase chain reaction (qPCR) was utilized to validate RNAseq findings as it a sensitive and reproducible method of quantifying and comparing relative gene expression. miScript Primer assay kits associated with the placental miRNA of interest were purchased through Qiagen. All primers were resuspended in 500uL of 1x TE (Tris-EDTA) Buffer and stored at -20°C. qPCR protocol was performed in a cDNA sterile hood using SYBR Green Master Mix. Master mix was a 1:3 ratio of universal primer, designated miScript primer, and SYBR Green Reagent. 5uL of master mix were

pipetted into individual wells in a 96 well plate. This was followed by 5uL of cDNA aliquots of each placental sample into each subsequent well for total volume of 10uL, with each sample performed in duplicate. Following centrifugation at 3000rpm for 4 minutes, the plate was immediately run through qPCR due to the SYBR green's light sensitivity. qPCR settings were calibrated according to both SYBR green and machine protocol. Annealing settings were set to: denaturation at 95°C for 15 minutes, 40 cycles of amplification at 95°C 15 sec, 55°C for 30 seconds and 70°C for 30 seconds, followed by 95°C for 5 seconds and 65°C for 1 minute prior to melting curve analysis.

Statistical analyses

Cohort analysis (pre-qPCR)

Elementary statistics were performed on multiple variables of the maternal-placental-fetal triad samples, those of interest being variables of known importance and/or measures of nutrient availability and sequestering between mother and fetus. Student T testing was performed to evaluate significant differences across these variables between the self-identified maternal racial groups. All numerical data points were scaled with logarithmic normalization prior to statistical analysis. P values < 0.05 were considered significant.

Placental miRNA gene expression analysis and correlations

Delta-delta cycle threshold (**ΔΔCt**) method was used to calculate gene fold expression, specifically by comparing normalized gene of interest expression relative to a standard housekeeping gene. This method allowed for the interpretation of the size and direction of gene expression in different conditions. Several reference genes were used

for housekeeping normalization, including the commonly used RNU6[50]. Additionally, sno68 and sno95 housekeeping genes were used to ensure reproducible results when testing miRNA expression in placental tissue [76]. Geometric Mean Excel analysis determined that best2 was an optimal normalizer to use for standardization of reference housekeeping gene[77]. ΔΔCt analysis values were normalized by logarithmic scaling prior to graphing relational data of gene expression. Dot plot graphs created in R were used to graphically display expression value in a manner to confirm expression relationships across maternal races.

Pearson correlation was used to evaluate relationships between placental miRNA expression and maternal-fetal variables, specifically IR (determined by Homeostatic Model Assessment of Insulin Resistance (**HOMA-IR**) [a calculated measure of pancreatic β cell fucntions and insulin resistance], placental weight and neonatal body composition measures (fat mass, percent fat mass and lean body mass). Correlational data was plotted with best fit line applied using R software. Multilinear regression, also in R software, was applied to adjust for possible interaction variables such as: maternal age, parity, pre-pregnancy BMI, smoking history, placental weight and neonatal sex. All numerical data points were normalized prior to statistical analysis. P values < 0.05 were considered significant.

PART 2. Supplementary RNAseq

Sample Selection

An additional 40 samples were selected for analysis to increase computing power, 20 from each racial group and balanced for BMI based on initial group sampling.

Selection of this supplementary cohort was determined by 2 standard deviations of the mean maternal age, gestational age and percent fat mass values of the initial group. In this group we also aimed to select primarily nonsmoking mothers, but ultimately, we selected several smokers in this cohort to better balance the number of smoking mothers in each of the ethnic and adiposity groups. Additionally, the cohorts were chosen to have similarly balanced ratios of male to female offspring to minimize any variation that could be attributed to larger male neonates. These samples were processed and sent for RNA sequencing and analysis, but sequencing results were not received by the time of thesis submission.

Sample preparation

RNA extraction

Frozen placental tissue (50 mg) was homogenized in 500uL Trizol Reagent, for the solubilization of biological material and denaturation of protein matter. Following tissue homogenization, 100uL of 100% chloroform was added to each sample to allow for phase separation between the DNA, RNA and protein. Samples were subsequently vortexed and incubated at room temperature for 10 minutes. After incubation, samples were centrifuged at 12,000g for 10 minutes at 4°C prior to aqueous layer collection and transferred into RNAse free vials, effectively separating the desired RNA from the other substances in the organic and interface layers. RNA was then precipitated as above with 250uL of 100% isopropanol from the aqueous phase, and RNA was pelleted by centrifugation at 12,000 revolutions/minute for 10 minutes at 4°C. After discarding the supernatant layer, pellets were washed twice with 0.5 ml 75% ethanol (diluted in RNase

free water) to eliminate any remaining contaminants from prior precipitations, vortexed and centrifuged as above. Following a 20-25 minute air-drying period, the samples were resuspended in 100uL of RNase free water and stored at -80°C until bioanalysis verification.

RNA bioanalysis

To ensure adequate RNA sample quality and to measure the concentration of isolated RNA prior to sequencing, all samples were assessed on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano (Chip) Kit for minimal measures of 160 ng/uL concentration and RIN >6.5 as recommended by the sequencing facility (methods part 1, page 15).

RNA ladder prep

The RNA ladder was pre-prepared by denaturing the samples at 70°C for 2 minutes followed by immediate cooling. The samples were stored in RNase free aliquots at -80°C until use.

Cleaning/setup

The Agilent 2100 Analyzer was decontaminated according to the model procedure with each chip run. 350uL of RNase ZAP was loaded into the designated cleaning plate, placed in the Analyzer for 1 minute followed by the repeated steps using 350uL of RNase free water. Following both cleaning steps, the Analyzer was left open to air dry for 10-15 seconds and then closed during chip and sample preparation. Following vortexing and spinning down of the RNA 6000 Nano dye to ensure adequate mixing and uniform solution concentration, 1uL of dye was added to a 65uL filtered gel aliquot

followed by vortexing and subsequently spun down for 10 minutes at 14000 rpm. The sample was stored in a light free environment until use to prevent degradation of the light sensitive dye. Using a new RNA Nano chip for each run, 9uL of the gel-dye mix was dispensed into the appropriate well. During dispensing, specific care was used to dispense solution at the bottom of the well without formation of a bubble to ensure adequate gel formation for accurate results. The chip was loading onto The Priming Station and subsequently closed to create a seal as the gel hardened. Once the gel settled (30 seconds), the clip was released, and the syringe was first manually guided up and then allowed to naturally spring back to its original location; to prevent disturbance of the gel. After opening the chip priming station, 9uL of the remaining gel-dye mixture were added to 2 additional marked wells in the chip.

Loading Marker and Samples for Bioanalysis

5uL of RNA 6000 Nano marker were injected into the remaining wells of the chip plate, into both the sample wells and designated ladder well. Prior to loading the ladder into its well, the sample was heat denatured at 70°C for 2 minutes to minimize secondary structure binding. 1uL of RNA ladder was loaded into its designated well, utilizing similar technique as above with the gel-dye mix. 1uL of each sample was then accordingly loaded into a single well per a predetermined layout. After all wells were loaded, the chip was placed in a horizontal adapted on the IKA-Model MS3Vortex Mixer and vortexed at 2400rpm for 1 minute for solution settlement in each well. After vortexing, the chip plate was oriented and loaded into the Aligent 2100 Analyzer and run in accordance with RNA analysis settings of the 2100 Expert Software.

Sequencing

Samples were submitted for RNA sequencing at Génome Québec Innovation Centre in accordance to their Illumina sequencing protocol for RNAseq. As of 01/30/20, all 40 samples were approved by quality measures at the Centre and were sent for processing. As of 3/16/20, laboratory functions were halted and later indefinitely suspended in the ongoing COVID-19 pandemic to preserve PPE and limiting community exposure. Data analysis with qPCR validation and statistical analysis is planned for future study as mentioned in Part 1 of the methods.

RESULTS

Part 1: Secondary analysis of initial RNAseq data

RNAseq was performed on 40 placenta samples, and qPCR was used to validate placental miRNAs of interest using 118 placenta samples from the original biobank of 508 placentas. These 118 mother-placental-fetal triads were selected based on having complete data sets and were balanced for maternal BMI and infant adiposity. They included smoking and non-smoking mothers from both racial groups as described below (Table 1). Maternal and neonatal variables analyzed were those which significantly affect nutrient availability and placental efficiency (maternal) and measures that correlate with fetal nutrient uptake. Maternal and neonatal variables were further separated and analyzed by maternal self-identified race, with significant differences between NHB and NHW women denoted by * (Table 1). Of the variables listed in Table 1, only maternal age, parity and maternal pre-pregnancy BMI (**ppBMI**) were significantly different between NHB and NHW women. Chi-square analysis did not detect a significant difference of neonatal sex distribution between racial groups ($p=0.053$). P values < 0.05 were considered significant.

	All N=118	NHB N=45	NHW N= 73
Maternal			
Maternal Age *(years)*	28.9 ± 6.3	26.4 ± 5.9	30.5 ± 6.1 *
Absolute Parity	1.6 ± 0.9	2 ± 1	1.4 ± 0.6 *
Smoking *(% smokers)*	21%	20%	22%
Maternal ppBMI (kg/m2)	27.8 ± 6.4	30.7 ± 6.2	26.1 ± 5.8 *
Placental Weight *(g)*	652 ± 177	625 ± 147	669 ± 193
Maternal HOMA-IR	3.0 ± 1.7	3.4 ± 2.0	2.7 ± 1.4
Neonatal			
Sex (*females: males)*	54 : 64	15 : 30	39 : 34

Gestational Age *(weeks)*	38.9 ± 0.6	38.8 ± 0.7	38.9 ± 0.5
Birth Weight (*kg*)	3.3 ± 0.5	3.2 ± 0.5	3.3 ± 0.6
Ponderal Index (g/cm3)	2.8 ± 0.4	2.8 ± 0.5	2.8 ± 0.3
FM *(kg)*	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
LBM *(kg)*	2.8 ± 0.4	2.8 ± 0.4	2.9 ± 0.4
% FM *(skinfolds)*	12.1 ± 4.3	11.7 ± 4.4	12.3 ± 4.3

Table 1 (continued): Cohort demographics of initial RNAseq. Significant p values from Student's T-testing between racial groups are denoted p <0.05*.

RNAseq analysis yielded information of relative expression levels of placental tissue miRNAs. Several miRNAs were notable for high relative expressions (> 6): hsa-miRNA-34b-3p, hsa-miRNA-34c-5p, hsa-miRNA-181d-5p, hsa-miRNA-192-5p (Table 2). Based on RNAseq analysis, NHB women had relatively higher expressions of placental miRNA-34b-3p, miRNA 34c-5p, and miRNA-181d-5p. Meanwhile miRNA-192-5p gene expression was greater in NHW women. These placental miRNAs, when run through TargetScan software (the predictive resource of finding biological gene targets in miRNAs)[74], were found to target several insulin-signaling or metabolism pathway-related genes (Table 3).

miRNA	**NHB relative expression**	**NHW relative expression**
hsa-miRNA-34b-3p	6.6	6.02
hsa- miRNA -34c-5p	11.5	10.9
hsa- miRNA -181d-5p	9.4	9.2
hsa- miRNA -192-5p	12.4	12.5

Table 2. **Highly expressed miRNAs in placentas of NHB and NHW women.** Relative expression levels of miRNA in NHB and NHW women [hsa denotes species (homo sapiens)].

Placental miRNA	Gene targets of interest	Gene functional pathways
hsa-miRNA-34b-3p	insulin induced gene 1[1], Insulin receptor [2], insulin like growth factor 1[3], Insulin substrate 1 [4], Insulin like growth factor 2 receptor [5]	Glucose hemostasis [1], Activation of insulin signaling and receptor recycling [2], ghrelin secretion and synthesis [3], insulin signaling in adipocytes [4], Degradation of IGF2 [5]
hsa-miRNA-34c-5p	solute carrier family 2 member 13[1], glucose 6 phosphatase catalytic 3[2], Phosphoglucomutase 1[3]	Small molecule transporter[1], catalytic unit of final enzymatic step of gluconeogenic and glycogenolytic pathways[2], glucose and glycogen metabolism[3]
hsa-miRNA-181d-5p	Heparan sulfate 3O sulfotransferase 3A1 [1], serum/ glucocorticoid regulated kinase family member 3 [2], solute carrier family 2 member 14 [3]	Integral membrane protein crucial in normotension (correlated with birth weight) [1], PI3K/Akt signaling [2], hexose GLUT transporter [3]
hsa-miRNA-192-5p	**Glucagon** like peptide 1 receptor[1], solute carrier family 25 member[2], mitochondrial phosphoenol-pyruvate carboxykinase 2[3]	Glucose-induced insulin secretion and signaling cascade[1], malate-aspartate shuttle[2], gluconeogenesis and adipogensis[3]

Table 3. Placental miRNAs of interest and their target genes. Shown are notable genes related to nutrient metabolism (specifically glucose) and/or insulin signaling pathways.

The placental expression of these miRNAs of interest (Table 2 and 3) were validated in a larger sample set (N=118, described in Table 1) by qPCR and ΔΔCt analysis. Placental miRNA-192-5p tended to be more highly expressed in NHW women as compared to their NHB peers. Placental miRNA-192-5p expression was visualized using dot plots, as seen in Figure 7. The placental miRNA-192-5p gene expression was not statistically different between NHW and NHB women (p=0.09).

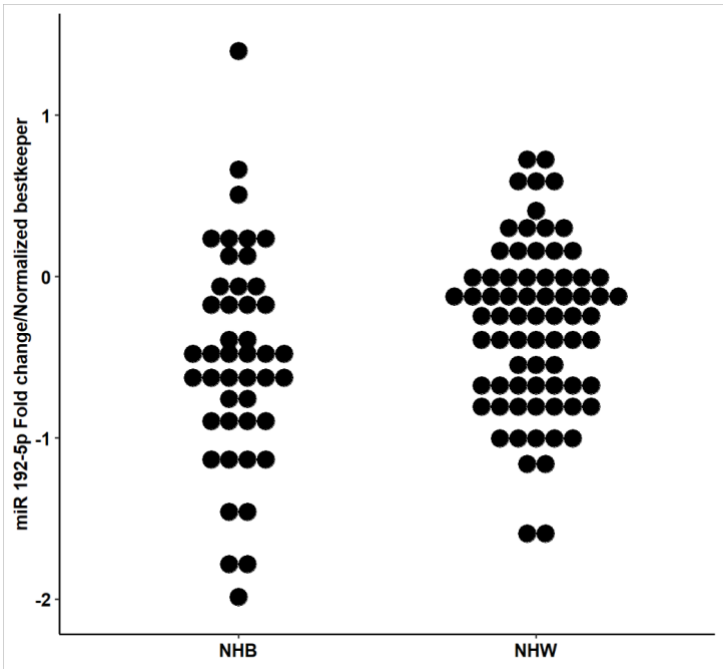

Figure 7: Placental miRNA-192-5p gene expression dot plot by race.

Pearson correlational analysis yielded no significant relationship between placental miRNA-192-5p expression and maternal insulin resistance as measured using HOMA-IR in NHW or NHB women. However, placental miR192-5p expression was positively correlated with lean body mass [**LBM**] (r = 0.344; p= 0.02), total fat mass [**FM**] (r = 0.406; p= 0.006) and percent fat mass [**%FM**] (r =0.374; p=0.01) in NHB women (Figures 8-10, respectively). There were no significant relationships between miRNA-192-5p and these measures of neonatal body composition in NHW women.

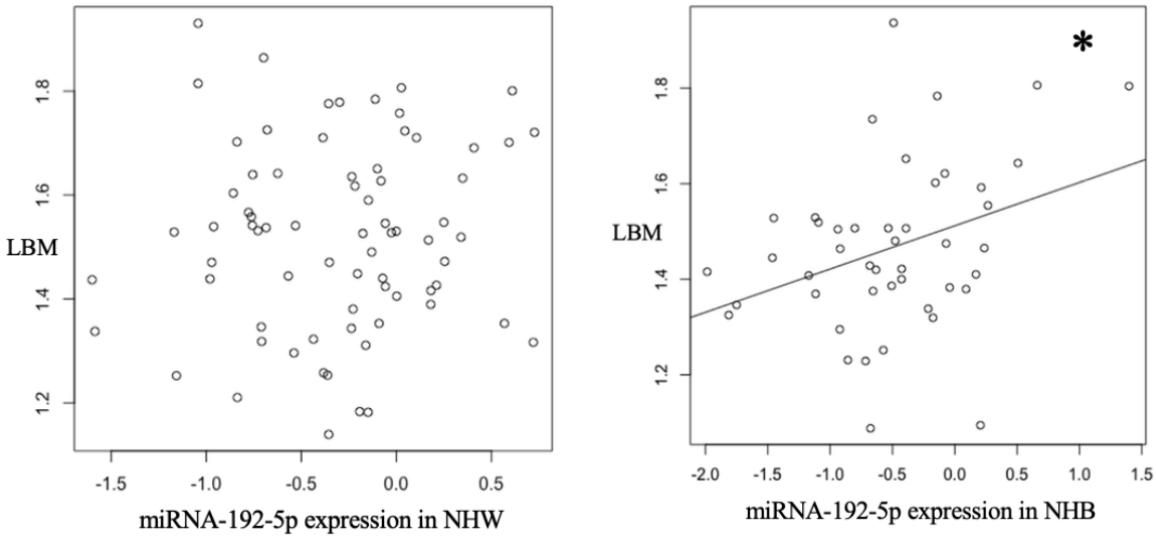

Figure 8. Relationship between placental miRNA-192-5p expression and neonatal lean body mass. Correlations of placental miRNA-192-5p gene expression to log-normalized neonatal LBM in NHW (left) and NHB women (right). Significant correlation between miRNA expression and LBM in NHB women only. P values <0.05 denoted with *.

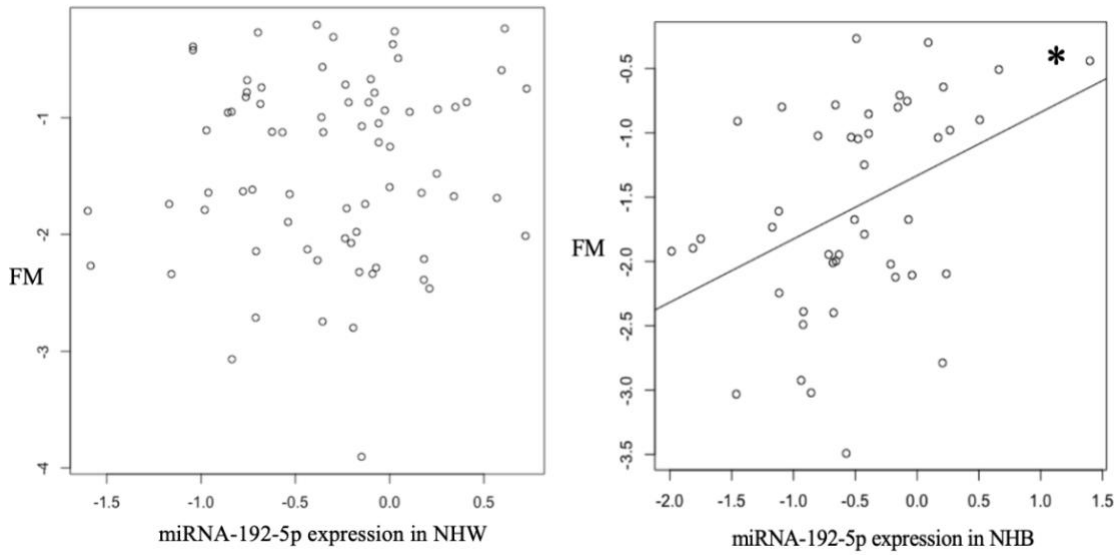

Figure 9. Relationship between placental miRNA-192-5p expression and neonatal fat mass. Correlations of placental miRNA-192-5p gene expression to log-normalized neonatal FM in NHW (left) and NHB women (right). Significant correlation between miRNA expression and FM in NHB women only. P values <0.05 denoted with *.

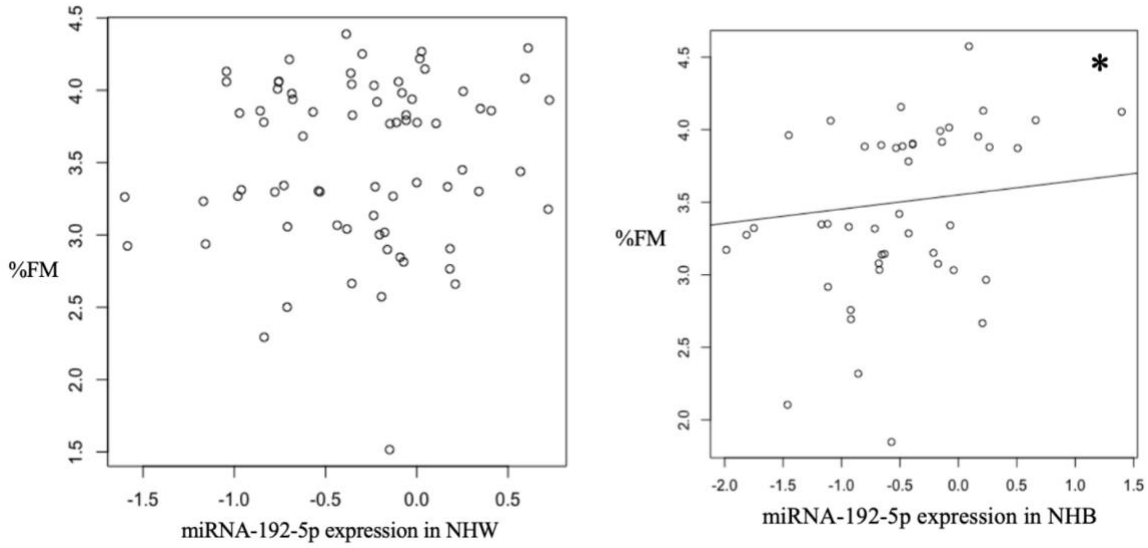

Figure 10. Relationship between placental miRNA-192-5p expression and neonatal % fat mass. Correlation of placental miRNA-192-5p gene expression to log-normalized neonatal FM in NHW (left) and NHB women (right). Significant correlation between miRNA expression and %FM in NHB women only. P values <0.05 denoted with *.

When adjusting for factors such as neontal sex and maternal smoking, HOMA-IR was not correlated with placental miRNA-192-5p gene expression in either NHB or NHW women. Additional adjustments for maternal age, parity and ppBMI (measures which were significantly different between racial groups in Table 1), did not alter the relationship between placental miRNA-192-5pm and maternal insulin resistance. Placental miRNA-192-5p expression was positively associated with maternal ppBMI in NHW women only (β coefficient =0.587; p=0.002) in simple linear regression. There was no significant relationship between placental weight and placental miRNA-192-5p expression in either NHB or NHW women.

NHB women had higher normalized fold changes of miRNA-34c-5p than NHW women, with statistically different gene expression levels between the two groups (p=1.74e-5), validating the RNAseq data presented in Table 2 (Figure 11).

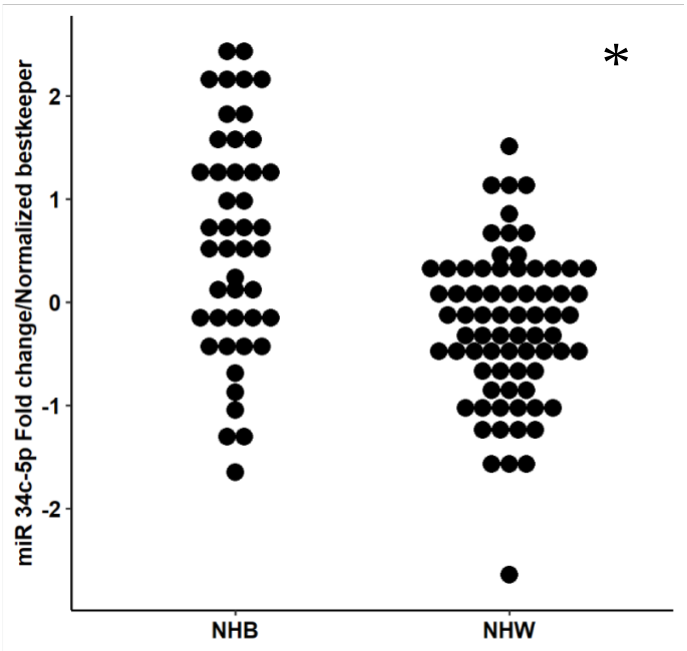

Figure 11: Placental miRNA-34c-5p gene expression dot plot based on race. P values <0.05 denoted with *.

There was no significant relationship between maternal HOMA-IR and placental miRNA-34c-5p gene expression in either NHW or NHB women, nor between miRNA-34c-5p expression and neonatal LBM in either group. In NHB women, miRNA-34c-5p expression was correlated with neonatal fat mass (r = 0.314; p=0.035) (Figure 12). This relationship was not signifant in NHW women. Similarly, the expression of miRNA-34c-5p was significantly corrleated to the %FM in infants of NHB women (r =0.305; p=0.04), but not in those born to NHW women (Figure 13).

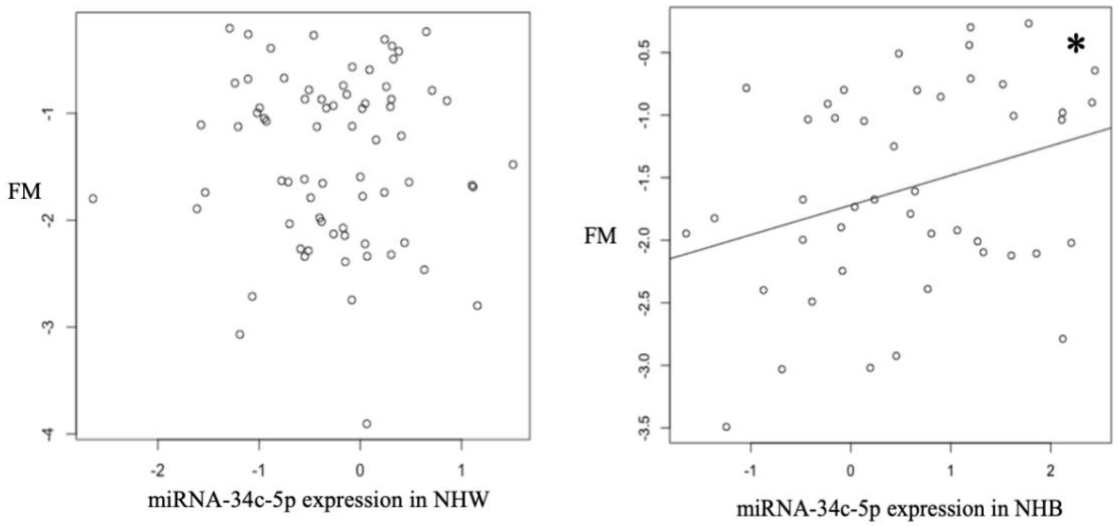

Figure 12. Relationship between placental miRNA-34c-5p expression and neonatal fat mass. Correlations of placental miRNA-34c-5p gene expression to log-normalized neonatal FM in NHW (left) and NHB women (right). Significant correlation between miRNA expression and FM in NHB women only. P values <0.05 denoted with *.

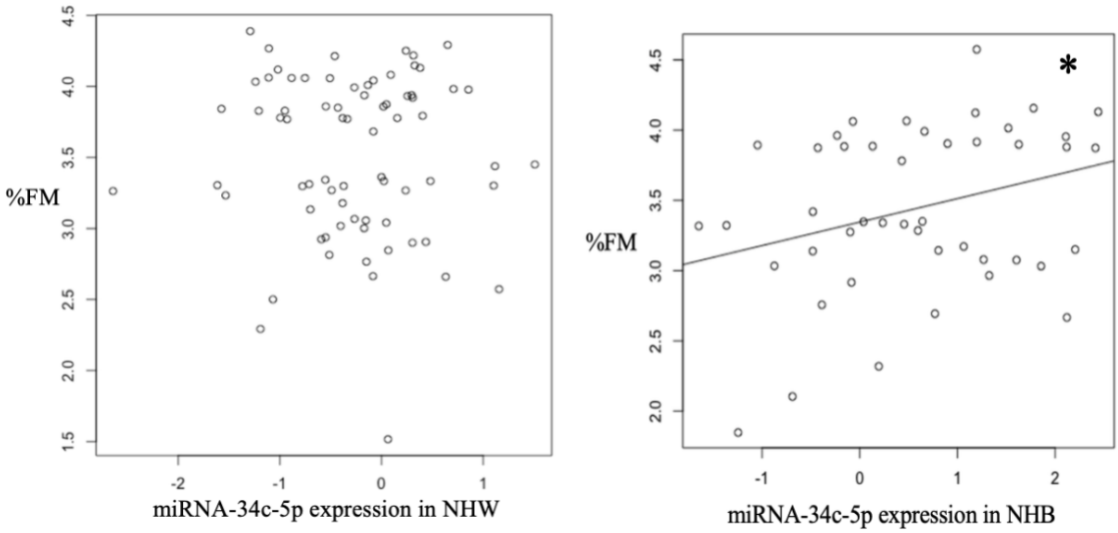

Figure 13. Relationship between placental miRNA-34c-5p expression and neonatal % fat mass. Correlations of placental miRNA-34c-5p gene expression to log-normalized neonatal %FM in NHW (left) and NHB women (right). Significant correlation between miRNA expression and FM in NHB women only. P values <0.05 denoted with *.

As with miRNA-192-5p, HOMA-IR was not correleted with placental miRNA-34c-5p gene expression in either racial group when adjusting for neontal sex and maternal smoking history. Further adjustment for maternal age, parity and ppBMI, HOMA-IR did not change this relationship. Maternal ppBMI in NHW women, but not NHB women, was positively associated with placental miRNA-34c-5p expression (β coefficient=0.91; p= 0.0008). There was no significant relationship between placental weight and miRNA-34c-5p expression in either NHB or NHW women as with miRNA-192-5p analysis.

Part 2: Supplementary RNAseq

As described in the methods, an additional 40 placental samples were selected for RNA sequencing to increase our statistical power to detect differences in placental miRNA expression between NHW and NHB women. Samples for this supplementary cohort were selected if they were within two standard deviations of the mean maternal age, gestational age and %FM values of the initial RNAseq cohort. Maternal and neonatal variables analyzed were those which significantly affect nutrient availability and placental efficiency (maternal) and measures of fetal nutrient uptake (neonatal). Table 4 shows the supplemental cohort demographics by race as well as combined with initial samples (N total=80). Maternal age was significantly different between NHW and NHB women (p=0.036), as was parity between the two groups (p=0.048). All neonatal measures, in particular those of neonatal body composition, were similar between NHB and NHW women. Neonatal sex was not different between groups (via chi-squared analysis). All nominal data was logarithmically normalized prior to statistical analysis. P values < 0.05 were considered significant.

	All N=80	NHB N=20	NHW N= 20
Maternal			
Maternal Age *(years)*	27.9 ± 5.5	25.5 ± 4.2	29.6 ± 5.9 *
Absolute Parity	1.5 ± 1.0	1.8 ± 1.1	1.3 ± 0.6 *
Smoking *(% smokers)*	17.5%	10%	20%
Placental Weight *(g)*	649 ± 190	628 ± 158	654 ± 104
Maternal HOMA-IR	3.0 ± 1.5	3.0 ± 2.0	3.0 ± 1.4
Neonatal			
Sex (*females: males)*	37 : 43	7 : 13	10 : 10
Gestational Age *(weeks)*	39 ± 1	39 ± 0	39 ± 0
Birth Weight (*kg*)	3.3 ± 0.6	3.2 ± 0.6	3.3 ± 0.4
Ponderal Index (g/cm3)	2.8 ± 0.3	2.7 ± 0.3	2.8 ± 0.2
FM *(kg)*	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
LBM *(kg)*	2.9 ± 0.4	2.9 ± 0.4	2.9 ± 0.3
% FM *(skinfolds)*	12.2 ± 4.4	11.5 ± 3.8	12.3 ± 2.9

Table 4: Cohort demographics of supplementary RNAseq. Cohort breakdowns divided by racial groupings, and also the combined total with initial RNAseq samples in column 'All'. Significant p values from Student's T-testing between racial groups are denoted $p < 0.05$*.

DISCUSSION

Part 1: Initial RNAseq

Upon analysis of the demographic breakdown of the 118 samples evaluated by qPCR, there was notable differences in maternal demographics. Specifically, NHB mothers were younger, had more prior births, and had greater ppBMI than their NHW counterparts. It is known that increased maternal age and absolute parity have both been associated with larger neonatal birth weights [78–80]. And although mothers who are multiparous are more likely to be older, larger birth weights were prevalent in infants born to older mothers when adjusting for parity [78]. This proves pertinent to our cohort analysis, as those two variables are related but not necessarily directly dependent. The lack of significant differences in neonatal body composition measures between races despite the differences in several maternal measures supports the hypothesis that there may be a physiological difference in nutrient availability and insulin sensitivity/ resistance between NHB and NHW women.

Understanding the differences between the comparison groups examined can help draw more accurate conclusions from the cohorts. The uneven distribution of male to female neonates in the NHB group is important to note, as males tend to grow larger and faster *in utero* than females [81] but are also at an increased risk of undernutrition due to less placental reserve capacity[82]. A larger sample size would prove beneficial for computing power. Similarly, the greater number of smokers in the NHW group is representative of the general trend of increased smoking in NHW mothers compared to NHB women [67,68], but can potentially shift neonatal growth measures since tobacco use

during pregnancy is negatively associated with fetal growth rate, weight and body composition[68]. While it has been reported that maternal obesity is positively correlated with birthweight and adiposity at birth [7], newer research favors a sexual dimorphism model, where maternal obesity is associated the greater female adiposity only [13]. With these differences in factors, we hypothesized that the neonates of NHB mothers would be, smaller in relation to their birth weights and/or body compositions. Selection of even ratios of females to males, preferential selection of nonsmoking mothers and more similar maternal demographics will be crucial for further analysis of the cohort to understand how miRNAs impact fetal growth. Despite both validated miRNAs having had relatively high expression levels in RNAseq but a lack of significant relationship between their expression and IR may point to the fact that these specific miRNAs are not crucial in modulating maternal IR in pregnancy. Further targeted validation of the miRNAs identified during the RNAseq dataset is necessary to understand possible implications of maternal insulin resistance and neonatal body composition outcomes.

Our secondary analysis of the RNAseq dataset indicated that in NHB women, miRNA-34b-3p, miRNA-34c-5p and miRNA-181d-5p were more highly expressed than in NHW women. Targeted qPCR validation of a larger sample set of both NHB and NHW women confirmed these findings in miRNA- 34c-5p and the trend in miRNA-192-5p. It is worth noting that when using ΔΔCt method to calculate normalized fold change, several placental housekeeping reference genes were used in initial calculations [76,83,84]. While RNU6 has been used in as a reference gene in placental research, comparative research in other organs shows it has a less stable expression [76]. Therefore, it is not

recommended as a first line reference gene when evaluating for differential miRNA expression. Bestkeeper method was therefore used to determine the 'best2' reference gene calculations[77] (geometric mean of sno68 and sno95), and were then carried through in expression analysis and graphics in this thesis and future work. However, the observed patterns in placental miRNA expression when calculating with best2 method were also consistent across multiple housekeeping genes (RNU6, sno68 and sno95). For future study, best2 method will continue to be used for reference gene because RNU6 housekeeper has differential expression in placental miRNAs of women of varying adiposities (manuscript pending). While both miRNA-34c-5p and miRNA-192-5p had differential expression across race, only miRNA-34c-5p expression was significantly different between NHB and NHW women.

While HOMA-IR (an indicator of maternal insulin resistance) was not significantly correlated with miRNA-192-5p or miRNA-34c-5p expression in either NHB or NHW women analyzed, both placental miRNAs had notable positive correlations with several neonatal body composition measures in NHB women. These findings may indicate that the above miRNAs may play a differential role in NHB pregnancies downstream of maternal insulin signaling or by a mechanism of placental efficiency which would affect nutrient availability to the fetus. This in turn may contribute to the observed and unexpected similarity in neonatal body composition factors between NHB and NHW groups despite statistically different maternal ages, absolute parities and ppBMIs. Maternal pre pregnancy BMI had a stronger positive effect on HOMA-IR in NHW women in multilinear regression analysis. And while ppBMI had a positive effect

on miRNA-192-5p expression in NHW women, ppBMI only had a positive effect on miRNA-34c-5p expression in NHB women. HOMA-IR was not significantly correlated with either 34c-5p or 192-5p miRNA expressions when adjusting for maternal age, parity and ppBMI. These findings do not exclude maternal characteristics playing a role in placental miRNA expression, which in turn could affect fetal adiposity.

Placental miRNAs may not play a direct or equal role in overall neonatal body composition in women of different races, given the multiple maternal and placental factors that can affect fetal growth. Placental miRNA-34c-5p's target genes included a solute carrier involved in nutrient transport and miRNA-192-5p genes included a protein involved in adipogenesis and the malate-aspartate shuttle. Both of these miRNAs could act downstream of insulin resistance, potentially altering nutrient transport in the placenta or fetal metabolism. Our findings may also be compounded or altered by the aforementioned social health disparities noted across racial groups. Increasing sampling size for RNAseq analysis and further analysis of other miRNAs would be useful to understand the proposed connection between placental miRNA expression and maternal insulin resistance as well as fetal growth.

Part 2: Supplementary RNAseq

Future studies include RNAseq analysis of the additional 40 samples, 20 in each racial group. This will not only double the dataset of samples sequenced, but potentially identify additional miRNAs that can be validated with qPCR. Similar to the relationships already assessed, we plan to perform further regression modeling between miRNA expression levels and insulin resistance measures. Other correlational testing to be done

with increased sample sizes for greater statistical power could include comparisons based on maternal and/or fetal adiposity and factors such as maternal weight gain during pregnancy. Following notable patterns observed, future steps could include metabolic pathway analysis of the miRNAs of interest. Utilizing miRNA-mimics to test the direct metabolic effects in placental tissue (and/or placental cell lines) would be critical to determine their placental-specific effects. Study of miRNA-induced maternal metabolic effects would require the use of female myocytes from pregnant and nonpregnant subjects. Trophoblast cells would be used to study placental changes, while neonatal myocytes/embryonic stem cells would be necessary to study downstream effects of miRNAs beyond placental efficiency or maternal metabolism changes. These experiments would aid in assessing the miRNA effects in fetal growth as well as metabolism and nutrient transport within the placenta.

CONCLUSION

While the two placental miRNAs (192-5p and 34c-5p) evaluated by targeted qPCR did not have significant impacts on HOMA-IR in either NHW or NHB women, the differences in impact of factors such as ppBMI, placental weight and neonatal body composition are important to note. This is important as it could inform changes to obstetric clinical care and in turn neonatal outcomes. Within the time constraints of seven months of research in addition to limitations due to the COVID-19 pandemic ceasing laboratory functions, more placental miRNA validation is planned based on Part 1 RNAseq data (specifically miRNA-34b-3p and miRNA-181d-5p). This work coupled with the additional statistical power from the supplementary RNA sequencing data (Part 2) will provide greater insight into the effects of placental miRNAs on maternal pregnancy adaptions such as insulin resistance as well as fetal growth and neonatal adiposity. Following validation of placental miRNAs of interest, next steps will include gene mapping of metabolic pathways based on target genes noted above and with the use of miRNA mimics to determine downstream effects in affected tissue types.

LIST OF JOURNAL ABBREVIATIONS

Am J Clin Nutr. – American Journal of Clinical Nutrition
Am J Epiemiol. – American Journal of Epidemiology
Am J Hum Bio – American Journal of Human Biology
Am J Obstet Gynecol. – American Journal of Obstetrics and Gynecology
Am J Public Health – American Journal of Public Health
Arch Dis Child – Archives of Disease in Childhood
Biochimica et Biophysica Acta – BBA: Biochimica et Biophysica Acta
Biol Reprod. – Biology of Reproduction
Biotechnol Lett. – Biotechnological Letters
BMJ – British Medical Journal
Curr Genomics – Current Genomics
Front Endocrinol – Frontiers in Endocrinology
Front Physiol. – Frontiers in Physiology
Int J Clin Endocrinol Metab. – International Journal of Endocrinology and Metabolism
Int J Dev Biol. – International Journal of Developmental Biology
Int J Gynaecol Obstet – International Journal of Gynaecology and Obstetrics
J Biomed Res – Journal of Biomedical Research
J Cell Sci. – Journal of Cell Science
J Clin Diagn Res. – JCDR: Journal of Clinical and Diagnostic Research
J Natl Med Assoc. – Journal of the National Medical Association
J Pediatr Endocrinol Metab. – Journal of Pediatric Endocrinology and Metabolism
JAMA – Journal of the American Medical Association
Matern Child Health – Maternal and Child Health Journal
Mol Hum Reprod. – Molecular Human Reproduction
Obstet Gynecol. – Obstetrics and Gynecology
Rev Obstet Gynecol. – Reviews in Obstetrics and Gynecology
Stem Cells Dev. – Stem Cells and Development
Trans Am Clin Climatol Assoc. – Transactions of the American Clinical and Climatological Association

REFERENCES

1. Fryar CD, Carroll MD, Ogden CL. *Prevalence of Overweight and Obesity Among Children and Adolescents Aged 2–19 Years: United States, 1963–1965 Through 2013–2014*. National Center for Health Statistics; 2016. https://www.cdc.gov/nchs/data/hestat/obesity_child_13_14/obesity_child_13_14.pdf. Accessed March 10, 2020.

2. Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. *The Lancet*. 2011;378(9793):815-825. doi:10.1016/S0140-6736(11)60814-3

3. Eriksson JG, Forsén T, Tuomilehto J, Osmond C, Barker DJ. Early growth and coronary heart disease in later life: longitudinal study. *BMJ*. 2001;322(7292):949-953. doi:10.1136/bmj.322.7292.949

4. Committee on Practice Bulletins—Obstetrics. Macrosomia: ACOG Practice Bulletin, Number 216. *Obstet Gynecol*. 2020;135(1):e18-e35. doi:10.1097/AOG.0000000000003606

5. Chiavaroli V, Derraik JGB, Hofman PL, Cutfield WS. Born Large for Gestational Age: Bigger Is Not Always Better. *The Journal of Pediatrics*. 2016;170:307-311. doi:10.1016/j.jpeds.2015.11.043

6. Gu S, An X, Fang L, et al. Risk factors and long-term health consequences of macrosomia: a prospective study in Jiangsu Province, China. *J Biomed Res*. 2012;26(4):235-240. doi:10.7555/JBR.26.20120037

7. Leddy MA, Power ML, Schulkin J. The Impact of Maternal Obesity on Maternal and Fetal Health. *Rev Obstet Gynecol*. 2008;1(4):170-178.

8. Mendola P, Mumford SL, Männistö TI, Holston A, Reddy UM, Laughon SK. Controlled direct effects of preeclampsia on neonatal health after accounting for mediation by preterm birth. *Epidemiology*. 2015;26(1):17-26. doi:10.1097/EDE.0000000000000213

9. Catalano PM. Trying to understand gestational diabetes. *Diabetic Medicine*. 2014;31(3):273-281. doi:10.1111/dme.12381

10. Kampmann U, Knorr S, Fuglsang J, Ovesen P. Determinants of Maternal Insulin Resistance during Pregnancy: An Updated Overview. Journal of Diabetes Research. doi:https://doi.org/10.1155/2019/5320156

11. Hay WW. Placental-Fetal Glucose Exchange and Fetal Glucose Metabolism. *Trans Am Clin Climatol Assoc*. 2006;117:321-340.

12. Catalano PM, Thomas AJ, Avallone DA, Amini SB. Anthropometric estimation of neonatal body composition. *Am J Obstet Gynecol*. 1995;173(4):1176-1181. doi:10.1016/0002-9378(95)91348-3

13. Mitanchez D, Jacqueminet S, Nizard J, et al. Effect of maternal obesity on birthweight and neonatal fat mass: A prospective clinical trial. *PLoS One*. 2017;12(7). doi:10.1371/journal.pone.0181307

14. O'tierney-ginn P, Presley MsL, Minium MsJ, Hauguel deMouzon S, Catalano P. Sex-specific effects of maternal anthropometrics on body composition at birth. *Am J Obstet Gynecol*. 2014;211(3):292.e1-292.e9. doi:10.1016/j.ajog.2014.05.031

15. Mingrone G, Manco M, Valera Mora ME, et al. Influence of Maternal Obesity on Insulin Sensitivity and Secretion in Offspring. *Diabetes Care*. 2008;31(9):1872-1876. doi:10.2337/dc08-0432

16. Catalano PM, Farrell K, Thomas A, et al. Perinatal risk factors for childhood obesity and metabolic dysregulation. *Am J Clin Nutr*. 2009;90(5):1303-1313. doi:10.3945/ajcn.2008.27416

17. Cho NH, Shaw JE, Karuranga S, et al. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice*. 2018;138:271-281. doi:10.1016/j.diabres.2018.02.023

18. Physiology - 6th Edition. https://www.elsevier.com/books/physiology/unknown/978-0-323-47881-6. Accessed December 1, 2019.

19. Liu M, Wright J, Guo H, Xiong Y, Arvan P. Chapter Two - Proinsulin Entry and Transit Through the Endoplasmic Reticulum in Pancreatic Beta Cells. In: Litwack G, ed. *Vitamins & Hormones*. Vol 95. The Pancreatic Beta Cell. Academic Press; 2014:35-62. doi:10.1016/B978-0-12-800174-5.00002-8

20. Insulin Synthesis and Secretion. http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/pancreas/insulin.html. Accessed March 10, 2020.

21. Weiss M, Steiner DF, Philipson LH. Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships. In: Feingold KR, Anawalt B, Boyce A, et al., eds. *Endotext*. South Dartmouth (MA): MDText.com, Inc.; 2000. http://www.ncbi.nlm.nih.gov/books/NBK279029/. Accessed March 10, 2020.

22. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001;414(6865):799-806. doi:10.1038/414799a

23. Harrison's principles of internal medicine, 17th ed. https://search-proquest-com.ezproxy.bu.edu/docview/200142518?accountid=9676&rfr_id=info%3Axri%2Fsid%3Aprimo. Accessed March 10, 2020.

24. Catalano PM, Roman-Drago NM, Amini SB, Sims EA. Longitudinal changes in body composition and energy balance in lean women with normal and abnormal glucose tolerance during pregnancy. *Am J Obstet Gynecol*. 1998;179(1):156-165. doi:10.1016/s0002-9378(98)70267-4

25. Cianni GD, Miccoli R, Volpe L, Lencioni C, Prato SD. Intermediate metabolism in normal pregnancy and in gestational diabetes. *Diabetes/Metabolism Research and Reviews*. 2003;19(4):259-270. doi:10.1002/dmrr.390

26. Kirwan JP, Varastehpour A, Jing M, et al. Reversal of Insulin Resistance Postpartum Is Linked to Enhanced Skeletal Muscle Insulin Signaling. *Int J Clin Endocrinol Metab*. 2004;89(9):4678-4684. doi:10.1210/jc.2004-0749

27. Sonagra AD, Biradar SM, K. D, Murthy D.S. J. Normal Pregnancy- A State of Insulin Resistance. *J Clin Diagn Res*. 2014;8(11):CC01-CC03. doi:10.7860/JCDR/2014/10068.5081

28. Friedman JE, Ishizuka T, Shao J, Huston L, Highman T, Catalano P. Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. *Diabetes*. 1999;48(9):1807-1814. doi:10.2337/diabetes.48.9.1807

29. Sivan E, Homko CJ, Chen X, Reece EA, Boden G. Effect of insulin on fat metabolism during and after normal pregnancy. *Diabetes*. 1999;48(4):834-838. doi:10.2337/diabetes.48.4.834

30. Kjos SL, Buchanan TA, Greenspoon JS, Montoro M, Bernstein GS, Mestman JH. Gestational diabetes mellitus: the prevalence of glucose intolerance and diabetes mellitus in the first two months post partum. *Am J Obstet Gynecol*. 1990;163(1 Pt 1):93-98. doi:10.1016/s0002-9378(11)90676-0

31. McIntyre HD, Chang AM, Callaway LK, et al. Hormonal and metabolic factors associated with variations in insulin sensitivity in human pregnancy. *Diabetes Care*. 2010;33(2):356-360. doi:10.2337/dc09-1196

32. Waters T, Minium J, Hagiac M, et al. 28: Does maternal insulin sensitivity improve immediately after delivery or do we need to wait until six weeks postpartum? *American Journal of Obstetrics and Gynecology*. 2015;212(1, Supplement):S20-S21. doi:10.1016/j.ajog.2014.10.074

33. Blackburn S. *Maternal, Fetal, & Neonatal Physiology: A Clinical Perspective*. 5th Edition. St. Louis, Missouri: Elsevier; 2017.

34. Hertig AT. The Human Placenta. *JAMA*. 1970;214(3):601-601. doi:10.1001/jama.1970.03180030117041

35. Benirschke K, Driscoll SG. The Pathology of the Human Placenta. In: Strauss F, ed. *Placenta*. Vol 5. Handbuch der Speziellen Pathologischen Anatomie und Histologie. Berlin, Heidelberg: Springer; 1967:97-571. doi:10.1007/978-3-662-38455-8_2

36. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. *Thrombosis Research*. 2004;114(5):397-407. doi:10.1016/j.thromres.2004.06.038

37. Wang Y, Zhao S. *Structure of the Placenta*. San Rafael, CA: Morgan & Claypool Life Sciences; 2010. https://www.ncbi.nlm.nih.gov/books/NBK53256/. Accessed March 10, 2020.

38. Carlson B. *Human Embryology and Developmental Biology*. 4th Edition. Philadelphia, PA: Mosby; 2008. https://www.elsevier.com/books/human-embryology-and-developmental-biology/carlson/978-0-323-05385-3. Accessed March 10, 2020.

39. SIU SOM Histology ERG. http://www.siumed.edu/~dking2/erg/placenta.htm. Accessed March 10, 2020.

40. Carter AM. Evolution of Placental Function in Mammals: The Molecular Basis of Gas and Nutrient Transfer, Hormone Secretion, and Immune Responses | Physiological Reviews. American Physiological Society. https://journals.physiology.org/doi/full/10.1152/physrev.00040.2011. Published October 1, 2012. Accessed March 10, 2020.

41. Loke YW, King A. *Human Implantation: Cell Biology and Immunology*. Cambridge University Press; 1995.

42. ILLSLEY NP, CANIGGIA I, ZAMUDIO S. Placental metabolic reprogramming: do changes in the mix of energy-generating substrates modulate fetal growth? *Int J Dev Biol*. 2010;54(0):409-419. doi:10.1387/ijdb.082798ni

43. Napso T, Yong HEJ, Lopez-Tello J, Sferruzzi-Perri AN. The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. *Front Physiol*. 2018;9:1091. doi:10.3389/fphys.2018.01091

44. Handwerger S, Freemark M. The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. *J Pediatr Endocrinol Metab*. 2000;13(4):343-356. doi:10.1515/jpem.2000.13.4.343

45. Brelje TC, Scharp DW, Lacy PE, et al. Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology*. 1993;132(2):879-887. doi:10.1210/endo.132.2.8425500

46. Barbour LA, McCurdy CE, Hernandez TL, Kirwan JP, Catalano PM, Friedman JE. Cellular Mechanisms for Insulin Resistance in Normal Pregnancy and Gestational Diabetes. *Diabetes Care*. 2007;30(Supplement 2):S112-S119. doi:10.2337/dc07-s202

47. Mouillet J-F, Ouyang Y, Coyne C, Sadovsky Y. MicroRNAs in placental health and disease. *Am J Obstet Gynecol*. 2015;213(4 0):S163-S172. doi:10.1016/j.ajog.2015.05.057

48. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol*. 2018;9. doi:10.3389/fendo.2018.00402

49. Wahid F, Shehzad A, Khan T, Kim YY. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2010;1803(11):1231-1243. doi:10.1016/j.bbamcr.2010.06.013

50. Ambros V, Bartel B, Bartel DP, et al. A uniform system for microRNA annotation. *RNA*. 2003;9(3):277-279. doi:10.1261/rna.2183803

51. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC Couples MicroRNA Biogenesis and Posttranscriptional Gene Silencing. *Cell*. 2005;123(4):631-640. doi:10.1016/j.cell.2005.10.022

52. Smith-Vikos T, Slack FJ. MicroRNAs and their roles in aging. *J Cell Sci*. 2012;125(1):7-17. doi:10.1242/jcs.099200

53. Huan T, Chen G, Liu C, et al. Age-associated microRNA expression in human peripheral blood is associated with all-cause mortality and age-related traits. *Aging Cell*. 2018;17(1). doi:10.1111/acel.12687

54. MacFarlane L-A, Murphy PR. MicroRNA: Biogenesis, Function and Role in Cancer. *Curr Genomics*. 2010;11(7):537-561. doi:10.2174/138920210793175895

55. Angueira AR, Ludvik AE, Reddy TE, Wicksteed B, Lowe WL, Layden BT. New Insights Into Gestational Glucose Metabolism: Lessons Learned From 21st Century Approaches. *Diabetes*. 2015;64(2):327-334. doi:10.2337/db14-0877

56. Mouillet J-F, Donker RB, Mishima T, Cronqvist T, Chu T, Sadovsky Y. The Unique Expression and Function of miR-424 in Human Placental Trophoblasts. *Biol Reprod*. 2013;89(2). doi:10.1095/biolreprod.113.110049

57. Donker RB, Mouillet JF, Chu T, et al. The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. *Mol Hum Reprod*. 2012;18(8):417-424. doi:10.1093/molehr/gas013

58. Xie L, Mouillet J-F, Chu T, et al. C19MC MicroRNAs Regulate the Migration of Human Trophoblasts. *Endocrinology*. 2014;155(12):4975-4985. doi:10.1210/en.2014-1501

59. Stadler B, Ivanovska I, Mehta K, et al. Characterization of microRNAs involved in embryonic stem cell states. *Stem Cells Dev*. 2010;19(7):935-950. doi:10.1089/scd.2009.0426

60. Department of Health, and Human Services. *Differences in Infant Mortality Between Blacks and Whites -- United States, 1980-1991*. Atlanta, GA: Centers for Disease Control and Prevention; 1994:288-289. https://www.cdc.gov/mmwr/preview/mmwrhtml/00030629.htm. Accessed March 10, 2020.

61. Wartko PD, Wong EY, Enquobahrie DA. Maternal birthplace is associated with low birth weight within racial/ethnic groups. *Matern Child Health J*. 2017;21(6):1358-1366. doi:10.1007/s10995-016-2241-4

62. Ramos GA, Caughey AB. The interrelationship between ethnicity and obesity on obstetric outcomes. *American Journal of Obstetrics and Gynecology*. 2005;193(3, Supplement):1089-1093. doi:10.1016/j.ajog.2005.06.040

63. Hales CM, Carroll MD, Fryar CD, Ogden CL. *Prevalence of Obesity Among Adults and Youth: United States, 2015–2016*. Hyattsville, MD: National Center for Health Statistics; 2017. https://www.cdc.gov/nchs/data/databriefs/db288.pdf. Accessed December 1, 2019.

64. Crawford S, Joshi N, Boulet SL, et al. Maternal Racial and Ethnic Disparities in Neonatal Birth Outcomes With and Without Assisted Reproduction. *Obstet Gynecol*. 2017;129(6):1022-1030. doi:10.1097/AOG.0000000000002031

65. Catov JM, Lee M, Roberts JM, Xu J, Simhan HN. Race Disparities and Decreasing Birth Weight: Are All Babies Getting Smaller? *Am J Epidemiol*. 2016;183(1):15-23. doi:10.1093/aje/kwv194

66. SINGH KA, HUSTON-PRESLEY LP, mencin P, Thomas A, AMINI SB, CATALANO PM. Birth Weight and Body Composition of Neonates Born to Caucasian Compared with African-American Mothers. *Obstet Gynecol*. 2010;115(5):998-1002. doi:10.1097/AOG.0b013e3181da901a

67. Perreira KM, Cortes KE. Race/Ethnicity and Nativity Differences in Alcohol and Tobacco Use During Pregnancy. *Am J Public Health*. 2006;96(9):1629-1636. doi:10.2105/AJPH.2004.056598

68. Drake P, Driscoll AK, Mathews T. *Cigarette Smoking During Pregnancy: United States, 2016*. National Center for Health Statistics; 2018. https://www.cdc.gov/nchs/data/databriefs/db305.pdf.

69. Bernstein IM, Mongeon JA, Badger GJ, Solomon L, Heil SH, Higgins ST. Maternal smoking and its association with birth weight. *Obstet Gynecol*. 2005;106(5 Pt 1):986-991. doi:10.1097/01.AOG.0000182580.78402.d2

70. Savitz DA, Dole N, Terry JW, Zhou H, Thorp JM. Smoking and Pregnancy Outcome among African-American and White Women in Central North Carolina. *Epidemiology*. 2001;12(6):636-642.

71. Jung E, Czajka-Narins D. Comparison of Growth of Black and White Infants During Their First Two Years of Life. *J Natl Med Assoc*. 1986;78(12):1157-1160.

72. Dauncey MJ, Gandy G, Gairdner D. Assessment of total body fat in infancy from skinfold thickness measurements. *Arch Dis Child*. 1977;52(3):223-227. doi:10.1136/adc.52.3.223

73. Cole PV, Hawkins LH, Roberts D. Smoking During Pregnancy and Its Effects on the Fetus. *BJOG: An International Journal of Obstetrics & Gynaecology*. 1972;79(9):782-787. doi:10.1111/j.1471-0528.1972.tb12920.x

74. TargetScanHuman 7.2. http://www.targetscan.org/vert_72/. Accessed March 11, 2020.

75. Home - Gene - NCBI. https://www.ncbi.nlm.nih.gov/gene. Accessed March 25, 2020.

76. Masè M, Grasso M, Avogaro L, et al. Selection of reference genes is critical for miRNA expression analysis in human cardiac tissue. A focus on atrial fibrillation. *Scientific Reports*. 2017;7(1):1-10. doi:10.1038/srep41127

77. Pfaffl MW, Tichopad A, Christian Prgomet, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnol Lett*. 2004;26(6):509-515. doi:10.1023/b:bile.0000019559.84305.47

78. Bakker R, Steegers E a. P, Biharie AA, Mackenbach JP, Hofman A, Jaddoe VWV. Explaining differences in birth outcomes in relation to maternal age: the Generation R Study. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2011;118(4):500-509. doi:10.1111/j.1471-0528.2010.02823.x

79. MacLeod S, Kiely JL. The effects of maternal age and parity on birthweight: a population-based study in New York City. *Int J Gynaecol Obstet*. 1988;26(1):11-19. doi:10.1016/0020-7292(88)90191-9

80. Murphy JF, Mulcahy R. The effect of age, parity, and cigarette smoking on baby weight. *Am J Obstet Gynecol*. 1971;111(1):22-25. doi:10.1016/0002-9378(71)90920-3

81. Misra DP, Salafia CM, Miller RK, Charles AK. Non-linear and gender-specific relationships among placental growth measures and the fetoplacental weight ratio. *Placenta*. 2009;30(12):1052-1057. doi:10.1016/j.placenta.2009.09.008

82. Eriksson JG, Kajantie E, Osmond C, Thornburg K, Barker DJP. Boys live dangerously in the womb. *Am J Hum Biol*. 2010;22(3):330-335. doi:10.1002/ajhb.20995

83. Mestdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biology*. 2009;10(6):R64. doi:10.1186/gb-2009-10-6-r64

84. Vidal DO, Ramão A, Pinheiro DG, et al. Highly expressed placental miRNAs control key biological processes in human cancer cell lines. *Oncotarget*. 2018;9(34):23554-23563. doi:10.18632/oncotarget.25264

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