

INVESTIGATING THE ROLE OF MATERNAL MITOCHONDRIA IN PREGNANCY:  
IMPLICATIONS FOR FETAL GROWTH AND GESTATIONAL LENGTH

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## ABSTRACT

**Background:** Little is understood about the role of mitochondria in pregnancy-related adaptations. Therefore, we evaluated associations of maternal early-to-mid pregnancy mitochondrial DNA copy number (mtDNAcn) and mtDNA methylation with birth size and gestational length.

**Methods:** Michigan women (n=396) provided venous bloodspots at median 11 weeks gestation to quantify mtDNAcn marker NADH-ubiquinone oxidoreductase chain 1 (*ND1*) using real-time quantitative PCR and mtDNA methylation at several regions within four mitochondria-specific genes using pyrosequencing: *MTTF* (mt-TF/RNR1 locus), *DLOOP* (D-loop promoter region, heavy strand), *CYTB* (cytochrome b), and *LDLR* (D-loop promoter region, light strand). We abstracted gestational length and birthweight from birth certificates and calculated birthweight z-scores using published references. We used multivariable linear regression to evaluate associations of mtDNAcn and mtDNA methylation with birthweight and birthweight z-scores. Cox Proportional Hazards Models (PHMs) and quantile regression characterized associations of mitochondrial measures with gestational length. We also considered differences by fetal sex.

**Results:** Using linear regression and Cox PHMs, mtDNAcn was not associated with birth outcomes, whereas associations of mtDNA methylation with birth outcomes were inconsistent. However, using quantile regression, mtDNAcn was associated with shorter gestation in female newborns at the upper quantiles of gestational length, but with longer gestational length in males at the lower quantiles of gestational length. Maternal *LDLR*, *DLOOP*, and *MTTF* methylation was associated with longer gestational length in females at the upper quantiles and in males at lower gestational length quantiles.

**Conclusions:** Maternal mtDNAcn and mtDNA methylation were associated with gestational length in babies born comparatively early or late, which could reflect adaptations in mitochondrial processes that regulate the length of gestation.

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## LIST OF ABBREVIATIONS

ARCH	Archive for Research on Child Health
CpG	Cytosine-phosphate-guanine
Ct	Threshold cycle
<i>CYTB</i>	Cytochrome b
<i>DLOOP</i>	D-loop promoter region
ENVIRONAGE	ENVIROnmental influence ON early AGEin
<i>LDLR</i>	D-loop promoter region, light strand
mtDNA	Mitochondrial DNA
mtDNAcn	Mitochondrial DNA copy number
<i>MTTF</i>	Mt-TF/RNR1 locus
<i>ND1</i>	NADH-ubiquinone oxidoreductase chain 1
ND1-F	<i>ND1</i> forward primer
ND1-R	<i>ND1</i> reverse primer
PCR	Polymerase chain reaction
PHM	Proportional hazards model
PM <sub>2.5</sub>	Particulate matter with aerodynamic diameter $\leq 2.5 \mu\text{m}$
qPCR	Quantitative polymerase chain reaction
S1	Location 1
S2	Location 2
SUMMIT	Supplementation with Multiple Micronutrients Intervention Trial
TMM	Tohoku Medical Megabank
WIC	Women, infant, and children

## CHAPTER 1: INTRODUCTION

Birthweight and gestational length are established predictors of child health, such that low birthweight (< 2500 g) and preterm birth (delivery < 37 weeks of gestation) are associated with neonatal mortality (1) and a range of morbidities, including neurological problems (2), intraventricular hemorrhage, necrotizing enterocolitis, and retinopathy (3-10). Beyond infancy, these outcomes are associated with higher risks of asthma (11), behavioral and emotional problems (12), and chronic metabolic conditions such as cardiovascular disease and type 2 diabetes mellitus (13). Similarly, macrosomia (birthweight > 4000 g) is associated with childhood and adulthood obesity (14-16). In the U.S., preterm birth is prevalent, being observed among one in every ten infants (17); furthermore, approximately 5-15% of infants are born small for gestational age (SGA; birthweight < 10<sup>th</sup> percentile for gestational age) and 5-20% of infants are born large for gestational age (LGA; birthweight > 90<sup>th</sup> percentile for gestational age) in developed countries (18, 19). Despite their lasting impact on the health of the individual, much remains to be understood about the biological mechanisms underlying length of gestation and size at birth.

Pregnancy requires adaptations of numerous maternal systems to provide sufficient energy for the placenta and the growing fetus. Mitochondria (independent organelles responsible for energy production and cellular respiration) are critical for cellular energy homeostasis (20) and are necessary for embryonic development and placental maintenance (21). Beyond energy generation, mitochondria are also responsible for converting cholesterol into sex-steroid hormones, including estrogen and progesterone (22, 23), both of which are essential to pregnancy progression. As such, mitochondrial function may be an important driver of pregnancy outcomes (21, 24). Mitochondrial DNA copy number (mtDNAcn) is a possible marker of mitochondrial function (25) that may be used to predict the roles of mitochondria in pregnancy. This is supported

by a study in mice demonstrating that maternal mitochondrial number per cell rapidly increased and remained elevated through most of pregnancy, in preparation for parturition (26). In humans, placental mtDNAcn has been associated with both extremes of the distribution of birth size. For instance, a case-control study in Spain reported that compared to placentas of 24 infants born AGA, placentas from 24 infants born SGA tended to have lower mtDNAcn (27), whereas a case-control study in China (n=54 in each group) reported that lower placental mtDNAcn was associated with higher odds of macrosomia (28). Because mtDNAcn has primarily been evaluated at the conclusion of pregnancy in placentas (29-31) and fetal cord blood (32-34), it is unclear whether these findings suggest that these changes are due to normal mitochondrial adaptation or the need to upregulate mitochondrial biogenesis due to insufficient energy production. Recently, a sub-analysis of randomly sampled women from the Supplementation with Multiple Micronutrients Intervention Trial (SUMMIT) observed that higher first and third trimester maternal mtDNAcn was associated with low birthweight (24), suggesting that maternal mtDNAcn may be a valuable tool for understanding the molecular underpinnings of pregnancy health. Thus, investigating links between early-pregnancy maternal mtDNAcn and birth outcomes warrants further investigation.

Beyond mtDNAcn, mtDNA methylation may also reflect mitochondrial function (35). Much of what we know about mtDNA methylation is derived from nuclear methylation, in which a methyl group is added to DNA residues to potentially alter the transcription of genes (36, 37). In non-pregnant populations, changes in mtDNA methylation are associated with several diseases, including Alzheimer's disease, type 2 diabetes, and amyotrophic lateral sclerosis (35, 36). Data on mtDNA methylation in pregnancy is limited, but it appears that environmental exposures may alter mtDNA methylation during this period. For example, in the U.S., maternal smoking status was positively associated with mtDNA methylation in both the placenta (n=96) and newborn foreskin



(n=62) (29). Similarly, one study of 381 pregnant Belgian women from the ENVIRONmental influence ON early AGEing (ENVIRONAGE) birth cohort reported that exposure to airborne particulate matter with aerodynamic diameter  $\leq 2.5 \mu\text{m}$  (PM<sub>2.5</sub>) was associated with higher placental mtDNA methylation (30). A proof-of-concept analysis in 60 women from ENVIRONAGE, selected based on perinatal exposure to tobacco and PM<sub>2.5</sub>, observed that placental mtDNA methylation was inversely associated with birthweight, regardless of maternal smoking status and PM<sub>2.5</sub> exposure (31). Despite its use in late pregnancy as a biomarker of toxicity from gestational exposure to environmental pollutants, no studies have considered the role of maternal mtDNA methylation in early pregnancy in relation to pregnancy outcomes.

Given the current limitations in the field, the primary objectives of the current study was to investigate whether maternal early-to-mid pregnancy mtDNAcn and mtDNA methylation are associated with birthweight and gestational length. Given the known differences in these outcomes by fetal sex (38, 39), we also considered whether associations between these mitochondrial markers and birth outcomes differed by fetal sex.

## CHAPTER 2: MATERIALS AND METHODS

### *2.1 Recruitment and enrollment of participants into the Archive for Research on Child Health (ARCH) pregnancy cohort and selection of the analytical sample*

The current study used data from a sub-sample of pregnant women from the Archive for Research on Child Health (ARCH), a prospective pregnancy cohort study based in Lansing, MI, with the overall goal of studying the effects of various prenatal factors on child health outcomes. ARCH was designed to be a low-cost and low participant-burden study that relied on archived information from birth certificates and medical records, brief survey interviews, and clinical specimens (extra tubes of blood and urine collected beyond routine purposes) (40, 41). The study has been described previously (41-43). Briefly, 801 women enrolled into the study at their first prenatal visit from one of three clinics in the Lansing area between 2008 and 2015; recruitment expanded in 2016 to include additional Michigan clinics and total enrollment reached 1042 women across all locations (40). Pregnant women that enrolled in ARCH underwent a study interview at their first prenatal care visit (cohort mean gestational age at enrollment was 13.4 weeks) (41). Eligible participants were  $\geq 18$  years of age and could reliably communicate in English. We collected additional information on demographics and socioeconomic status from women at study enrollment.

Maternal blood collection was added to the study protocol approximately six months after the commencement of initial recruitment and was thus only available for a subset of Lansing participants (n=590). Of these, 500 women had quantifiable DNA, and at least one measure of mtDNAcn or mtDNA methylation. We excluded women carrying multiples (n=7) from the analysis because we first must understand mitochondrial health and function in singleton pregnancies before investigating mitochondrial function in pregnancies with greater energy

demands. Derivation of the analytic sample is described in **Supplemental Figure 1**. The current analytic sample includes 396 women who had available data on at least one biomarker (mtDNAcn or mtDNA methylation), all birth outcomes (birthweight, birthweight z-score, and gestational length), and all covariates (see below). All participants provided written informed consent and the study was approved by the Institutional Review Boards of Michigan State University, the Michigan Department of Health and Human Services, and Sparrow Hospital.

## 2.2 *Collection of maternal sociodemographic and lifestyle information at enrollment*

At the first prenatal visit, women completed an intake questionnaire to provide information on relevant sociodemographic, lifestyle, and health characteristics such as age, race/ethnicity, annual household income, educational attainment, marital status, tobacco use before or during pregnancy, and pre-pregnancy height and weight. In the survey, women reported their ethnicity by answering “Hispanic or Latino” or “Not Hispanic or Latino” to the question “Your ethnic category: Hispanic or Latino”, and reported their race by answering “Yes” to one or a combination of “American Indian or Alaska Native”, “Black or African American”, “Native Hawaiian or Pacific Islander”, “Asian”, or “White” in response to the question “Your racial category (check all that apply)”. Women reported their annual household income as “Under \$25,000”, “\$25,000 to \$49,000”, “\$50,000 to \$74,999” and “\$75,000 or above”. To ascertain marital status, women responded “Married, living with baby’s father”, “Married”, “Unmarried, living with baby’s father”, or “Unmarried” to the question “What is your current marital status?”. Information on pregnancy complications, tobacco use, parity, and maternal pre-pregnancy weight and height were also abstracted from birth certificates.

We calculated pre-pregnancy body mass index (BMI, in  $\text{kg}/\text{m}^2$ ) from pre-pregnancy weight (lbs) and height (ft and in) collected via the intake survey or abstracted from the birth certificates.

To improve our sample size, we prioritized data abstracted from birth certificates when available. However, we used survey-reported pre-pregnancy weight when birth certificate values were missing (n=2) and when the difference between abstracted delivery and pre-pregnancy weights was over 100 pounds (n=4). Abstracted pre-pregnancy weight and height did not differ significantly from the survey-reported pre-pregnancy weight and height (**data not shown**).

### 2.3 *Maternal bloodspot collection and quantification of mtDNAcn*

At the first prenatal appointment, we collected bloodspots from a maternal venous sample. Specifically, up to five small (up to 0.5 cm<sup>2</sup> in size) spots of blood were blotted onto filter paper using a pipette and frozen at -80°C until processing. DNA was isolated from half of one full blood spot from each participant using the QIAamp DNA microkit (Qiagen), and a modified protocol for blood spots that included spin baskets in the microcentrifuge tubes after the proteinase digestion step and addition of the RNase digestion step. DNA concentration for each sample was quantified using Qubit 4 fluorometer (Invitrogen) with the Qubit dsDNA Broad Range Assay Kit (Invitrogen, Catalog number: Q32850) and diluted to 0.5 ng/μL before subsequent quantification of mtDNAcn using real-time quantitative polymerase chain reaction (qPCR) (44, 45). mtDNAcn was measured by quantification of a mitochondrial gene, NADH-ubiquinone oxidoreductase chain 1 (*ND1*). The primer sequences were the forward primer (ND1-F): 5'-CCCTAAAACCCGCCACATCT-3' and reverse primer (ND1-R): 5'-GAGCGATGGTGAGAGCTAAGGT-3', as described previously (46). The qPCR quantification of *ND1* was performed on 384-well plates by the QuantaStudio™ 6 Flex Real-time PCR system (Applied Biosystems) with a 10 μL reaction mixture containing 4.0 μL of PerfeCTa SYBR® Green qPCR Master MIX 2×, 4.0 μL of 0.5 ng/μL DNA, 1 μL of 5 μM ND1-forward primer, and 1 μL of 5 μM ND1-reverse primer. All reactions of each sample were carried out in triplicate. The qPCR was conducted following the conditions below: 2 min at 50°C,

10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The presence of a single polymerase chain reaction (PCR) product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s, then 65 °C for 15 s. The threshold cycle (Ct) data were analyzed and transformed using the standard curve method with the Quantastudio™ Real-Time PCR Software (version 1.3, Applied Biosystems, CA) using a 1:4 dilution curve starting at 10 ng/μL and ending at 0.15625 ng/μL.

#### 2.4 *Assessment of mtDNA methylation in maternal bloodspots*

DNA samples were shipped on dry ice to the University of Michigan and stored at -80°C until analysis. The samples were linearized using 10 U of BamHI per sample (Thermo Fisher Scientific) and bisulfite-treated using the Epitect 96 Bisulfite Kit (Qiagen). Each bisulfite reaction utilized 143 – 600 ng of DNA. PCR was performed using HotStarTaq Master Mix (Qiagen), forward primers, biotinylated reverse primers, and 2 μL bisulfite converted DNA. Four mitochondria-specific genes relevant to pregnancy health and disease susceptibility were selected and their sequences amplified: *MTTF* (mt-TF/RNR1 locus) (47), *DLOOP* (D-loop promoter region, heavy strand) (31), *CYTB* (cytochrome b gene region) (48), *LDLR* (D-loop promoter region, light strand) (31). Primers were designed using the PyroMark Assay Design Software 2.0 (Qiagen) based on assays previously described by others (31, 47, 48) (**Supplemental Table 1**).

DNA methylation at cytosine-phosphate-guanine dinucleotides (CpG sites) was analyzed using the PyroMark ID Pyrosequencer (Qiagen) for each of the four genes: *MTTF* (one CpG site at location one (S1) and two CpG sites at location two (S2)), *DLOOP* (3 CpG sites), *CYTB* (three CpG sites), and *LDLR* (three CpG sites). A small fraction of samples was duplicated (15%), with results averaged when both passed. Several controls were included to ensure quality: no-template PCR controls, 0% methylated human DNA, 50% methylated human DNA, and 100% methylated

human DNA. Additional internal quality control checks were performed by the Pyro Q-CpG software to confirm proper bisulfite conversion, adequate signal, and other measures. Analysis was performed only on samples passing all quality control checks.

## 2.5 *Collection of gestational length and birth weight data and calculation of birthweight z-scores*

We abstracted last menstrual period (LMP)-based and obstetric-based estimates of gestational length from birth certificates. To create the gestational length variable, we applied the algorithm developed by Basso and Wilcox (49, 50) to identify records with likely errors in gestational age estimation by LMP. In brief, if both the LMP- and obstetric-based estimates were available, we checked whether the difference was less than or equal to two weeks. In these cases, the birthweight z-score based on the LMP-based estimate was examined to see if it was considered plausible for that gestational age (49). If it was, the LMP-based estimate was retained. If the resulting z-score was not plausible and the birthweight z-score from the obstetric-based estimate was within the acceptable range, the obstetric-based estimate was used. If neither the LMP-based nor the obstetric-based estimate birthweight z-score was within range, we discarded the record. If the LMP-based and obstetric-based estimates differed by more than two weeks, the obstetric-based estimate was examined first and retained if the birthweight z-score was within range; if not, the LMP-based estimate was examined and retained if the birthweight z-score was within range. When only one estimate of gestational length was available (or within the 22–44-week window), we examined the birthweight z-score to see if it was within range and could be retained. Lastly, we applied criteria from Alexander et al. to identify additional implausible birthweights for gestational age, but none were observed (50, 51). No records were excluded, and LMP-based estimates were appropriate and used for n=307 participants, whereas obstetric-based estimates were used for n=89

participants in final analysis (**Supplemental Figure 2**). Our primary outcome was gestational length, which we evaluated continuously (days) and categorically as: delivery prior to 39 weeks (< 39 weeks) or delivery at or after 39 weeks ( $\geq 39$  weeks) gestation (52). Newborn birthweights were abstracted from birth certificates, and we calculated sex-specific birthweight-for-gestational age z-scores according to published methods using a U.S. population-based reference (50).

## 2.6 Statistical analysis

A maximum of 396 women were available for statistical analyses, but the sample size varied depending on the exposure of interest. Using the available literature, we evaluated several potential covariates to include in our statistical models and selected covariates *a priori*, including factors associated with mitochondrial biomarkers and birth outcomes, to generate a directed acyclic graph (DAG, **Supplemental Figure 3**) (27, 28, 53-56). We assessed correlations between all covariates to test for potential multicollinearities, but covariates were only weakly-to-moderately correlated ( $r < 0.35$ , **data not shown**). Additionally, we reviewed splines to help ascertain the appropriate operationalization of each covariate. Final covariate-adjusted statistical models included age, pre-pregnancy BMI, race/ethnicity, annual household income, tobacco use before and during pregnancy, parity, fetal sex, gestational age at bloodspot collection, and the nuclear gene human  $\beta$ -globin (*hBG*) or methylation plate number. Age, *hBG*, and gestational age at bloodspot collection were included as continuous variables, and the operationalization and corresponding reference groups of categorical covariates are delineated in **Table 1**. These covariates potentially represent latent constructs we cannot directly measure (57): structural racism (race/ethnicity), socioeconomic status, including neighborhood- and individual-level stressors (race/ethnicity, annual household income), lifestyle (tobacco use and pre-pregnancy BMI), reproductive health (parity and age), pregnancy characteristics (gestational age at bloodspot

collection and fetal sex), and laboratory environment (*hBG* and plate number).

### 2.6.2 Descriptive statistics

We reported the characteristics of the analytic sample as median (25<sup>th</sup>, 75<sup>th</sup> percentiles) for continuous variables or n (%) for categorical variables. Similarly, the median (25<sup>th</sup>, 75<sup>th</sup> percentiles), were reported for mtDNA biomarkers and birth outcomes. Additionally, we reported the percentage of women in the analytic sample with non-zero values of mtDNAcn and mtDNA methylation.

### 2.6.3 Primary analyses for birthweight and birthweight z-score

To accomplish our objective, we first applied linear regression models to evaluate associations of maternal mtDNAcn and mtDNA methylation with birthweight and birthweight z-scores. We considered whether associations differed by fetal sex by including an interaction term between each mtDNA biomarker and fetal sex. We natural log-transformed *NDI* and all CpG sites and their averages from *LDLR*, *DLOOP*, *CYTB*, and *MTTF S1* in linear regression models to improve model fit and interpretation. *MTTF S2* CpG sites and their average were not transformed. Several women had methylation equal to zero for some CpG sites, so we added a constant of 1 to all reported values using the equation  $[\ln(\text{mtDNA methylation} + 1)]$  to avoid undefined estimates during transformation. Birthweight and birthweight z-score were not transformed. The resulting  $\beta$ -estimates and 95% confidence intervals (CIs) were back-transformed using the equation  $\beta * \ln(2)$  and are interpreted as the change in birthweight (in grams) or birthweight z-score for every two-fold increase in mtDNAcn or mtDNA methylation. Where the mtDNA measure was not transformed, the resulting  $\beta$ -estimates and 95% CIs were interpreted as the change in birthweight (in grams) or birthweight z-score for each 1% increase in mtDNA methylation. In all analyses, we first evaluated unadjusted models and then covariate-adjusted models (refer to **section 2.6.1** for



the final covariate list) and checked regression diagnostics to ensure model assumptions were met.

#### 2.6.4 Primary analyses for gestational length

We took several approaches to understand whether mtDNAcn and mtDNA methylation in pregnancy were associated with gestational length, overall and by fetal sex. First, we applied Cox Proportional Hazards Models (PHMs). We selected Cox PHMs for this relationship because of its ability to effectively model the time-dependent nature of delivery, which linear regression cannot accomplish, and it may be a better predictor of the true association because its increased statistical power increases its effectiveness at modeling gestational length as a continuous variable, which logistic regression cannot provide (58, 59). In our Cox PHM analysis, we considered delivery < 39 weeks of gestation as our time-to-event outcome because of relatively low preterm birth rates in our analytic sample. Specifically, gestational length was fitted as the time scale and birth < 39 weeks was defined as the event. Births  $\geq 39$  weeks were treated as censored observations, thus assuming that the effects of mtDNAcn and mtDNA methylation on survival are constant over time while gestational length is less than 39 weeks (60). Given that earlier gestational ages at delivery are associated with higher risks of adverse birth outcomes, we selected birth < 39 weeks as our event of interest to better understand the potential impact of being born before 39 weeks (52, 61, 62). Similar to the birthweight analysis, we evaluated whether associations of mtDNAcn and mtDNA methylation differed by fetal sex by including a multiplicative interaction term between each mtDNA biomarker and fetal sex. As before, we natural log-transformed *ND1* and all CpG sites from *LDLR*, *DLOOP*, *CYTB*, and *MTTF S1* in PHMs to improve model fit and interpretation. *MTTF S2* CpG sites were not transformed. The resulting hazard ratios (HR) and 95% confidence intervals (CIs) for these analyses were back-transformed using  $e^{\ln(2) * \ln(HR)}$  and are interpreted as the percent change in the hazard of delivery < 39 weeks compared to birth  $\geq 39$  weeks for every

two-fold increase in mtDNAcn or mtDNA methylation. When back-transformation was not necessary, results were interpreted as the percent change in the hazard of birth < 39 weeks compared to birth  $\geq$  39 weeks for every one-unit increase in mtDNAcn or % mtDNA methylation. We evaluated unadjusted models and covariate-adjusted models.

Given the non-normal distribution of gestational length in our study, we conducted quantile regression analyses to further understand whether there is a non-linear association of mtDNAcn or mtDNA methylation with gestational age at delivery. We used quantile regression because it estimates non-normally distributed outcomes by detecting differences in the associations of the exposure across distributions of the outcome; for example, if the associations on the tails of the distributions are suspected to differ from the association at the median (63-65). We estimated confidence intervals using a rank-based approach (63-65). Results are presented at the 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, and 95<sup>th</sup> percentiles of gestational length (in days), back-transformed as defined for linear regression and are interpreted as the change, in days, in gestational length for every two-fold increase in mtDNAcn or mtDNA methylation (for transformed mtDNA methylation measures) or as the change in days in gestational length for each 1% increase in mtDNA methylation (for non-transformed mtDNA methylation measures) at each quantile. Quantile regression models accounted for covariates listed in **section 2.6.1**.

All statistical analyses were conducted using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). Cox PHM analyses were conducted using PROC PHREG, linear regression analyses were conducted using PROC GLM, and quantile regression was performed using PROC QUANTREG. Guided by recommendations from the American Statistical Association, we assessed patterns and magnitudes of association, as well as 95% CIs, to determine meaning and significance rather than considering *p*-values (66, 67). We did not adjust for multiple comparisons

(68).

#### 2.6.5 *Sensitivity analyses*

We conducted several sensitivity analyses to evaluate the robustness of the results. First, we specified preterm births (delivery before 37 weeks of gestation) as the censoring event in PHMs for the gestational length analysis to determine if observed associations were driven by preterm birth (**Supplemental Table 2**). Secondly, to account for potential biological complications that may encourage early labor or alter birthweight trajectory, we evaluated two additional models where we excluded women with birth certificate-abstracted gestational hypertension and gestational diabetes (**Supplemental Table 2, Supplemental Table 3, and Supplemental Table 4**). Because quantile regression requires a continuous outcome, and obstetric-based estimates are reported in weeks only (integers), we conducted one final sensitivity analysis where we added an imputed day to gestational weeks in women whose gestational length was determined using the obstetric estimate (n=89). This was accomplished by setting a seed and using the RAND function in SAS to generate a random integer between 0 and 6. We once again conducted quantile regression analyses overall and stratified by fetal sex to examine the robustness of our primary findings (**Supplemental Table 5, Supplemental Table 6, and Supplemental Table 7**).

## CHAPTER 3: RESULTS

### 3.1 *Characteristics of the analytic sample*

Characteristics of the 396 eligible ARCH participants are outlined in **Table 1**. The mothers in our study had a median age of 25 years (25<sup>th</sup>, 75<sup>th</sup> percentiles: 22, 29) and provided a bloodspot at median 11 (9, 14) weeks of gestation. Nearly half of the mothers were non-White (42%), and over two-thirds had an annual household income less than \$25,000 (68%), did not use tobacco during pregnancy (71%), and did not have obesity (71%). Almost half of the participants were nulliparous (40%) and carrying a male fetus (49%).

### 3.2 *Maternal mtDNA biomarker levels*

All women had non-zero levels of *ND1* (**Table 2**). In terms of mtDNA methylation, many women had above zero % methylation at an *LDLR* CpG site (18-87% methylation > zero), one *DLOOP* CpG site (31-60% methylation > zero), one *CYTB* CpG site (18-36% methylation > zero), or one *MTTF* S1 or S2 CpG site (30-89% methylation > zero) (**Table 2**). Levels of mtDNAcn and mtDNA methylation were largely similar between women carrying females and males (**Table 2**).

### 3.3 *Distributions of birthweight, birthweight z-score, and gestational length at birth*

The distributions of birthweight, birthweight z-score, and gestational length are reported in **Table 3**. Briefly, the median (25<sup>th</sup>, 75<sup>th</sup> percentile) birthweight, birthweight z-score, and gestational length were 3,345 grams (3,005, 3,643), -0.1 (-0.7, 0.6), and 276 days (269, 283), respectively. Nearly one-third of infants were born prior to 39 weeks (32%) and the prevalence of preterm birth was 8% (**Table 3**).

### 3.4 *Associations of mtDNAcn with birthweight, birthweight z-score, and gestational length*

In unadjusted and covariate-adjusted linear regression analyses, mtDNAcn was not associated with birthweight or birthweight z-score. Associations did not differ by fetal sex (**Figure**

**1, Supplemental Table 3, and Supplemental Table 4).**

We observed no overall or fetal sex-specific associations of mtDNAcn with gestational length using Cox PHM (**Figure 2A** and **Supplemental Table 2**). Similarly, mtDNAcn was not associated with overall gestational length using quantile regression (**Figure 2B** and **Table 7**); however, these associations differed by fetal sex. For instance, in females who were in the 95<sup>th</sup> percentile of gestational age (n=11), a two-fold increase in mtDNAcn was marginally associated with a 3.1-day (95% CI: -6.6, 0.5) shorter gestation (**Figure 2B** and **Table 8**). Conversely, in males who were at the 5<sup>th</sup> percentile of gestational length (n=9), a two-fold increase in mtDNAcn was associated with a 3.3-day (95% CI: 0.2, 6.5) longer gestation (**Figure 2B** and **Table 9**). In sensitivity analyses where we added a random day to obstetric-based estimates of gestation length, quantile regression models remained consistent; however, associations at lower and upper quantiles became stronger, primarily driven by males and females, respectively (**Supplemental Table 5, Supplemental Table 6, and Supplemental Table 7**).

### 3.5 *Associations of mtDNA methylation with birthweight, birthweight z-score, and gestational length at birth*

In unadjusted linear regression models, mtDNA methylation was not associated with birthweight or birthweight z-scores (**Supplemental Table 3** and **Supplemental Table 4**). When adjusted for covariates, a two-fold increase in *CYTB* CpG3 % methylation was associated with marginally higher birthweight ( $\beta$ : 66.5 grams, 95% CI: -9.7, 142.6) and birthweight z-score ( $\beta$ : 0.11, 95% CI: -0.03, 0.24). Additionally, a two-fold increase in *CYTB* CpG3 % methylation was associated with marginally higher birthweight ( $\beta$ : 67.9 grams, 95% CI: -17.9, 153.6) (**Table 4**). A two-fold increase in *LDLR* CpG2 % and *LDLR* CpG3 % methylation was associated with a 0.15 (95% CI: -0.04, 0.33) and 0.14 (95% CI: -0.01, 0.30) higher birthweight z-score, but not

birthweight (**Table 4** and **Table 5**). When considering differences in associations by fetal sex, the association of *CYTB* CpG3 % methylation with birth size was driven by males, such that a two-fold increase in % methylation was associated with a 105.3-gram (95% CI: -4.6, 215.2) higher birthweight and a 0.16 (95% CI: -0.03, 0.36) higher birthweight z-score (**Table 4** and **Table 5**). *LDLR* CpG2 methylation was associated with higher birthweight z-scores in females ( $\beta$ : 0.22, 95% CI: -0.02, 0.46) but not males (**Table 5**), and the association of *LDLR* CpG3 % methylation with birthweight z-score was driven by females ( $\beta$ : 0.19, 95% CI: 0.03, 0.42) (**Table 5**). Finally, a two-fold increase in *MTTF* S1 CpG1 % methylation was associated with a 143.2-gram (95% CI: 23.5, 262.9) higher birthweight and a 0.17 (95% CI: -0.04, 0.38) higher birthweight z-score in males, whereas a two-fold increase in the average % methylation of *MTTF* S2 CpG sites was associated with a 0.07-unit (95% CI: 0.01, 0.15) higher birthweight z-score in males (**Table 4** and **Table 5**).

Overall, mtDNA methylation was not associated with gestational length in crude or covariate-adjusted Cox PHMs (**Table 6** and **Supplemental Table 2**). However, when we considered differences in associations by fetal sex, in females, each two-fold increase in *DLOOP* CpG1 % methylation was associated with a 30% lower hazard (HR: 0.7, 95% CI: 0.4, 1.1) of birth before 39 weeks while *CYTB* CpG2 % methylation was associated with a 30% higher hazard (HR: 1.3, 95% CI: 1.0, 1.7; **Table 6**). Using quantile regression, mtDNA methylation was associated with gestational length at its lower (5<sup>th</sup>) and higher (75<sup>th</sup> and 95<sup>th</sup>) percentiles (**Table 7**), which differed by fetal sex. Specifically, mtDNA methylation of several CpG sites was associated with longer gestation in females at the upper (50<sup>th</sup>, 75<sup>th</sup>, and 95<sup>th</sup>) percentiles (**Table 8**). In males, mtDNA methylation of most CpG sites was generally associated with longer gestation in males in the lower (5<sup>th</sup>, 25<sup>th</sup>) percentiles, and shorter gestation at the highest (95<sup>th</sup>) percentile (**Table 9**). In sensitivity analyses where we added a random day to obstetric-based estimates of gestation length,

results from quantile regression models were generally consistent, but with more precision and generally stronger associations in the upper quantiles in males and females (**Supplemental Table 5, Supplemental Table 6, and Supplemental Table 7**).

## CHAPTER 4: DISCUSSION

### 4.1 *Summary of major findings*

In a sample of Midwestern U.S. women with relatively lower socioeconomic status, several measures of mitochondrial function were associated with gestational length, and less so with size at birth. Importantly, associations of mtDNAcn or mtDNA methylation with birth outcomes differed by fetal sex and additionally depended on whether babies were born relatively earlier or later. Taken together, these findings suggest that mitochondrial adaptations may support birth size and the length of gestation in some babies. However, additional experimental studies are needed to establish the causal nature of our findings.

### 4.2 *Mitochondrial measures and birth size*

In our study, maternal early-to-mid pregnancy mtDNAcn was not associated with birthweight or birthweight z-score, which is not in line with prior studies (24, 69). For instance, in pregnant Japanese women from the Tohoku Medical Megabank Project (TMM; n=149), maternal early-to-mid pregnancy mtDNAcn (as determined using the average of the difference between (Ct value of *SLCO2B1* – Ct value of *ND1*) and (Ct value of *SERPINA1*– Ct value of *ND5*), was inversely associated with birthweight in males but not in females (69, 70). Similarly, a study in 520 pregnant women from the SUMMIT in Lombok reported that gestational mtDNAcn was negatively associated with birthweight, strongest when considering third trimester mtDNAcn (24). Contrarily, a pooled analysis of placentas from Spanish (n=376) and Belgian (n=550) participants enrolled in the INfancia y Medio Ambiente (INMA) and the ENVIRONAGE cohorts reported that each interquartile range increase in placental mtDNAcn was associated with a 140.2-g increase in birthweight, which was primarily driven by males (71). Beyond birthweight, several small case-control studies have reported associations of maternal peripheral mtDNA content (72) and



placental mtDNA content (73, 74) with intrauterine growth restriction (IUGR), such that higher mtDNAcn or mtDNA content was associated with higher odds of an IUGR pregnancy. Comparatively, cross-sectional case-control studies reported placental mtDNA content was associated with lower odds of SGA delivery in Spanish women (n=24 SGA cases and n=24 controls) (27) and higher odds of both SGA and LGA delivery in Argentinian women (n=17 SGA cases, n=14 LGA cases, and n=57 controls) (75). Discrepancies between these studies and ours may be attributable to differences in the timing of sample collection for mtDNAcn quantification, tissue, methods used to determine mtDNAcn and the gestational length, the population of interest, and covariates included in statistical models. For instance, the median timepoint of blood collection in our study was 11 weeks gestation, whereas some studies collected blood samples much later, such as the second and third trimesters or at birth using the placenta. This is a notable difference, given that some studies have reported increases or decreases in maternal mtDNAcn across trimesters (72, 76). Additionally, in studies that measured mtDNA in placenta, it may be difficult to identify a temporal relationship with birth outcomes (as compared to our study and others that assessed maternal mtDNA long before drastic increases in fetal growth or parturition). Importantly, our study differed from others as we isolated DNA from maternal blood spots rather than from circulating blood or placenta. Although this does not influence the interpretation of our mtDNA methylation results, our mtDNAcn findings may need to be interpreted with some prior context. Specifically, because we could not quantify the volume of blood loaded on each bloodspot, we diluted all isolated DNA samples to have a consistent amount of measured DNA prior to qRT-PCR, meaning that our mtDNAcn results can be interpreted as the number of mitochondrial DNA copies within the context of total DNA (since each reaction presumably had a consistent number of total DNA copies). Other studies that isolate circulating DNA also

normalize their reaction to total DNA by measuring a housekeeping (genomic DNA) transcript, so it is likely that our methods are quite alike, but additional studies may be needed to compare the two approaches. Overall, given the numerous other methodological differences in prior studies, more evidence is needed to establish mtDNAcn as a reliable predictor of fetal growth.

While we did not observe associations of mtDNAcn with birthweight or birthweight z-score, methylation of several CpG sites was sex-specifically associated with higher birthweight and birthweight z-scores. Although current epidemiologic studies have rarely considered the role of maternal mtDNA methylation in fetal growth, several studies investigating whether placental mtDNA methylation is associated with birth outcomes have reported inconsistent findings. For example, in pregnant women from Belgium (n=255 non-smokers, n=65 former-smokers, and n=62 smokers) enrolled in the ENVIRONAGE cohort, placental methylation of the nuclear DNA gene *CYP11A1* was lower in women who currently smoked, and newborns whose mothers were current smokers had lower birthweight (30). A follow-up case-control study in the same cohort evaluated methylation of *DLOOP* and *LDLR* from 60 placentas and reported that methylation of *LDLR* was not associated with birthweight, whereas *DLOOP* methylation was negatively associated with birthweight (31). Similar to our findings, additional studies have also reported associations of placental and maternal mtDNA methylation with birth size, particularly increased odds of macrosomia and LGA (28, 77). Given the paucity of experimental evidence and conflicting epidemiologic findings, the exact biological mechanisms driving the observed associations are unknown. Additionally, due to the plethora of functions performed by the mitochondria – including synthesis of sex-steroid hormones critical to pregnancy, further studies are needed to elucidate underlying mechanisms that are impacted by mtDNA methylation and further evaluate its capacity as a driver or biomarker of fetal growth.

#### 4.3 *mtDNA methylation and mtDNAcn are associated with gestational length*

In our study, maternal mtDNAcn was positively associated with gestational length in infants delivered comparatively earlier or later (lower or upper quantiles of gestation length). The observed relations were sex-specific, such that the association in the lower quantiles of gestational length was driven by males and the association at the upper percentiles was driven by females. Unlike our study, the TMM Project observed that mtDNAcn was associated with shorter gestational length overall, and in both females and males (69). There is a scarcity of literature evaluating maternal mtDNAcn in relation to length of gestation, and few studies have investigated whether mtDNA content is reflective of pregnancy health. In one study, investigators coalesced five placental RNA-sequencing datasets from Australian women to characterize changes in mitochondria-related transcripts throughout pregnancy and then conducted a case-control study using placentas from preterm (n=8), preterm fetal growth restriction (n=8), term (n=8), and post-term (n=7) births to understand how expression of 13 regulatory genes was associated with gestational length (78). Overall, mitochondria-related gene expression was elevated in placentas of women who delivered preterm and post-term, though findings were inconsistent across cohorts (78). There are several reasons for discrepancies between our findings and the current literature, including sample size, study design, origin country, biospecimen source and timing, and mtDNAcn assessment methodology (as discussed above). Our findings suggest that women who deliver earlier or later may possess a different mitochondrial phenotype than women who deliver at term. As discussed previously, this either points to adaptations in mitochondrial mechanisms in women who go on to deliver earlier or later, or may highlight a potential causal target of earlier or later birth. Given the inconsistencies between our findings and the few other available studies, substantially more work is needed to characterize the role of mtDNAcn in gestational length.

Our study is one of the first to investigate associations of maternal mtDNA methylation with gestational length. We observed positive and negative gene- and sex-specific associations of maternal mtDNA methylation with gestational length. Importantly, we considered three methylation sites and their average to better characterize the association, and our findings suggest that CpG-specific methylation may be more predictive of gestational length. Prior experimental and human studies have not yet considered mtDNA methylation in the context of gestational age at delivery; however, given that gestational length is predictive of numerous child health outcomes, and the potential role of energy metabolism (and other mitochondrial processes like steroidogenesis, particularly the synthesis of estrogens and progestins) in determining gestational length, future studies should consider the role of mitochondrial methylation (and resulting gene expression and function) in pregnancy.

#### *4.4 Strengths and limitations*

Our study has some limitations, but several notable strengths. First, like many others, our study relied on birth certificates for maternal and infant outcomes, maternal demographics, and diagnosis of gestational diabetes and gestational hypertension. However, birth certificates are reliable and have been validated for pregnancy characteristics such as gestational length and birth weight (79, 80). Second, ARCH is comprised of women from a Midwest U.S. city and therefore our results may not be generalizable to other populations, but our analytic sample represented groups that are often under-studied, including non-White women and those from lower-income households. Third, mtDNAcn and mtDNA methylation were quantified from maternal peripheral blood in early pregnancy at one time point rather than across pregnancy, which limits the generalizability of our findings as we may miss important windows where mtDNA is more associated with birth outcomes. However, our biomarkers were quantified prior to collection of

birth outcomes, thus we were able to identify potentially temporal and biologically plausible associations. Fourth, we could not control for residual confounding, such as diet quality or physical activity, which may be important for mitochondrial function and pregnancy health. However, we did generate a DAG using the available literature and *a priori* considerations. Fifth, mtDNA methylation is difficult to quantify and the true presence and function of mtDNA methylation is debated (36, 81). Despite that, our study utilized a novel method and successfully quantified methylation at several locations. Sixth, we acknowledge the risk of type 1 error that may be increased due to the number of analyses we performed; however, we aimed to reduce the risk by evaluating trends across models to determine associations and conducting additional analyses when non-linear associations were suspected. Finally, because of the paucity of studies investigating the association between maternal mitochondrial function and pregnancy outcomes, we are limited in our capacity to compare our findings to the literature. However, we added potentially important observations by considering differences in fetal sex, a known key factor in fetal growth trajectory and overall gestational health.

## CHAPTER 5: CONCLUSIONS

In Midwestern U.S. women with relatively low socioeconomic status, we observed that measures of mitochondrial function were primarily positively associated with parameters of fetal growth and gestational length, with few exceptions. Our findings also suggest that the impact of mtDNA methylation on birth outcomes may differ by fetal sex and the specific location of methylation. Given the importance of proper fetal growth and gestation for acute and lifelong health, future studies are warranted to corroborate our findings to further elucidate the role of mtDNA<sub>cn</sub> and mtDNA in various mitochondria-mediated adaptations critical in pregnancy. This knowledge will contribute to a broader understanding of gestational mitochondrial adaptations and may support changes to clinical practice that consider mitochondrial endpoints as potential early biomarkers of fetal growth, helping to identify pregnancies that may be at risk.

## REFERENCES

1. Watkins WJ, Kotecha SJ, Kotecha S. All-Cause Mortality of Low Birthweight Infants in Infancy, Childhood, and Adolescence: Population Study of England and Wales. *PLoS Med.* 2016;13(5):e1002018. Epub 2016/05/11. doi: 10.1371/journal.pmed.1002018. PubMed PMID: 27163787; PMCID: PMC4862683.
2. Linsell L, Malouf R, Morris J, Kurinczuk JJ, Marlow N. Risk Factor Models for Neurodevelopmental Outcomes in Children Born Very Preterm or With Very Low Birth Weight: A Systematic Review of Methodology and Reporting. *Am J Epidemiol.* 2017;185(7):601-12. Epub 2017/03/25. doi: 10.1093/aje/kww135. PubMed PMID: 28338817.
3. Boyle EM, Poulsen G, Field DJ, Kurinczuk JJ, Wolke D, Alfirevic Z, Quigley MA. Effects of gestational age at birth on health outcomes at 3 and 5 years of age: population based cohort study. *BMJ.* 2012;344:e896. Epub 2012/03/03. doi: 10.1136/bmj.e896. PubMed PMID: 22381676; PMCID: PMC3291750 form at [www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.
4. Coathup V, Boyle E, Carson C, Johnson S, Kurinczuk JJ, Macfarlane A, Petrou S, Rivero-Arias O, Quigley MA. Gestational age and hospital admissions during childhood: population based, record linkage study in England (TIGAR study). *BMJ.* 2020;371:m4075. Epub 2020/11/27. doi: 10.1136/bmj.m4075. PubMed PMID: 33239272; PMCID: PMC7687266 at [www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) and declare: financial support from the MRC for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.
5. Klevebro S, Lundgren P, Hammar U, Smith LE, Bottai M, Domellof M, Lofqvist C, Hallberg B, Hellstrom A. Cohort study of growth patterns by gestational age in preterm infants developing morbidity. *BMJ Open.* 2016;6(11):e012872. Epub 2016/11/20. doi: 10.1136/bmjopen-2016-012872. PubMed PMID: 27856479; PMCID: PMC5128893.
6. Manuck TA, Rice MM, Bailit JL, Grobman WA, Reddy UM, Wapner RJ, Thorp JM, Caritis SN, Prasad M, Tita AT, Saade GR, Sorokin Y, Rouse DJ, Blackwell SC, Tolosa JE, Eunice Kennedy Shriver National Institute of Child H, Human Development Maternal-Fetal Medicine Units N. Preterm neonatal morbidity and mortality by gestational age: a contemporary cohort. *Am J Obstet Gynecol.* 2016;215(1):103 e1- e14. Epub 2016/01/17. doi: 10.1016/j.ajog.2016.01.004. PubMed PMID: 26772790; PMCID: PMC4921282.

7. Piccolo B, Marchignoli M, Pisani F. Intraventricular hemorrhage in preterm newborn: Predictors of mortality. *Acta Biomed.* 2022;93(2):e2022041. Epub 2022/05/13. doi: 10.23750/abm.v93i2.11187. PubMed PMID: 35546030; PMCID: PMC9171888 stock ownership, equity interest, patent/licensing arrangement, etc.) that might pose a conflict of interest in connection with the submitted article.
8. Wang YX, Ding M, Li Y, Wang L, Rich-Edwards JW, Florio AA, Manson JE, Chavarro JE. Birth weight and long-term risk of mortality among US men and women: Results from three prospective cohort studies. *Lancet Reg Health Am.* 2022;15. Epub 2023/01/13. doi: 10.1016/j.lana.2022.100344. PubMed PMID: 36632048; PMCID: PMC9830740.
9. Ward RM, Beachy JC. Neonatal complications following preterm birth. *BJOG.* 2003;110 Suppl 20:8-16. Epub 2003/05/24. doi: 10.1016/s1470-0328(03)00012-0. PubMed PMID: 12763105.
10. Wilcox AJ. On the importance--and the unimportance--of birthweight. *Int J Epidemiol.* 2001;30(6):1233-41. Epub 2002/02/01. doi: 10.1093/ije/30.6.1233. PubMed PMID: 11821313.
11. Sonnenschein-van der Voort AM, Arends LR, de Jongste JC, Annesi-Maesano I, Arshad SH, Barros H, Basterrechea M, Bisgaard H, Chatzi L, Corpeleijn E, Correia S, Craig LC, Devereux G, Dogaru C, Dostal M, Duchon K, Eggesbo M, van der Ent CK, Fantini MP, Forastiere F, Frey U, Gehring U, Gori D, van der Gughten AC, Hanke W, Henderson AJ, Heude B, Iniguez C, Inskip HM, Keil T, Kelleher CC, Kogevinas M, Kreiner-Moller E, Kuehni CE, Kupers LK, Lancz K, Larsen PS, Lau S, Ludvigsson J, Mommers M, Nybo Andersen AM, Palkovicova L, Pike KC, Pizzi C, Polanska K, Porta D, Richiardi L, Roberts G, Schmidt A, Sram RJ, Sunyer J, Thijs C, Torrent M, Viljoen K, Wijga AH, Vrijheid M, Jaddoe VW, Duijts L. Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. *J Allergy Clin Immunol.* 2014;133(5):1317-29. Epub 2014/02/18. doi: 10.1016/j.jaci.2013.12.1082. PubMed PMID: 24529685; PMCID: PMC4024198.
12. El Marroun H, Zeegers M, Steegers EA, van der Ende J, Schenk JJ, Hofman A, Jaddoe VW, Verhulst FC, Tiemeier H. Post-term birth and the risk of behavioural and emotional problems in early childhood. *Int J Epidemiol.* 2012;41(3):773-81. Epub 2012/05/04. doi: 10.1093/ije/dys043. PubMed PMID: 22552873.
13. Kerkhof GF, Willemsen RH, Leunissen RW, Breukhoven PE, Hokken-Koelega AC. Health profile of young adults born preterm: negative effects of rapid weight gain in early life. *J Clin Endocrinol Metab.* 2012;97(12):4498-506. Epub 2012/09/21. doi: 10.1210/jc.2012-1716. PubMed PMID: 22993033.



14. Mitchell EA, Stewart AW, Braithwaite I, Hancox RJ, Murphy R, Wall C, Beasley R, Group IPTS. Birth weight and subsequent body mass index in children: an international cross-sectional study. *Pediatr Obes*. 2017;12(4):280-5. Epub 2016/05/14. doi: 10.1111/ijpo.12138. PubMed PMID: 27170099.
15. Palatianou ME, Simos YV, Andronikou SK, Kiortsis DN. Long-term metabolic effects of high birth weight: a critical review of the literature. *Horm Metab Res*. 2014;46(13):911-20. Epub 2014/12/05. doi: 10.1055/s-0034-1395561. PubMed PMID: 25473824.
16. Zhao Y, Wang SF, Mu M, Sheng J. Birth weight and overweight/obesity in adults: a meta-analysis. *Eur J Pediatr*. 2012;171(12):1737-46. Epub 2012/03/03. doi: 10.1007/s00431-012-1701-0. PubMed PMID: 22383072.
17. Martin JA, Hamilton BE, Osterman MJ. Births in the United States, 2021. *NCHS Data Brief*. 2022(442):1-8. Epub 2022/09/01. PubMed PMID: 36043891.
18. Chiavaroli V, Castorani V, Guidone P, Derraik JG, Liberati M, Chiarelli F, Mohn A. Incidence of infants born small- and large-for-gestational-age in an Italian cohort over a 20-year period and associated risk factors. *Ital J Pediatr*. 2016;42:42. Epub 2016/04/28. doi: 10.1186/s13052-016-0254-7. PubMed PMID: 27117061; PMCID: PMC4845339.
19. Henriksen T. The macrosomic fetus: a challenge in current obstetrics. *Acta Obstet Gynecol Scand*. 2008;87(2):134-45. Epub 2008/01/31. doi: 10.1080/00016340801899289. PubMed PMID: 18231880.
20. Fisher JJ, Bartho LA, Perkins AV, Holland OJ. Placental mitochondria and reactive oxygen species in the physiology and pathophysiology of pregnancy. *Clin Exp Pharmacol Physiol*. 2020;47(1):176-84. Epub 2019/08/31. doi: 10.1111/1440-1681.13172. PubMed PMID: 31469913.
21. Rodriguez-Cano AM, Calzada-Mendoza CC, Estrada-Gutierrez G, Mendoza-Ortega JA, Perichart-Perera O. Nutrients, Mitochondrial Function, and Perinatal Health. *Nutrients*. 2020;12(7). Epub 2020/07/28. doi: 10.3390/nu12072166. PubMed PMID: 32708345; PMCID: PMC7401276.
22. Miller WL. Steroid hormone synthesis in mitochondria. *Mol Cell Endocrinol*. 2013;379(1-2):62-73. Epub 2013/05/01. doi: 10.1016/j.mce.2013.04.014. PubMed PMID: 23628605.
23. Papadopoulos V, Miller WL. Role of mitochondria in steroidogenesis. *Best Pract Res Clin Endocrinol Metab*. 2012;26(6):771-90. Epub 2012/11/22. doi: 10.1016/j.beem.2012.05.002. PubMed PMID: 23168279.

24. Priliani L, Febinia CA, Kamal B, Shankar AH, Malik SG. Increased mitochondrial DNA copy number in maternal peripheral blood is associated with low birth weight in Lombok, Indonesia. *Placenta*. 2018;70:1-3. Epub 2018/10/15. doi: 10.1016/j.placenta.2018.08.001. PubMed PMID: 30316321.
25. Castellani CA, Longchamps RJ, Sun J, Guallar E, Arking DE. Thinking outside the nucleus: Mitochondrial DNA copy number in health and disease. *Mitochondrion*. 2020;53:214-23. Epub 2020/06/17. doi: 10.1016/j.mito.2020.06.004. PubMed PMID: 32544465; PMCID: PMC7375936.
26. Bartho LA, Fisher JJ, Walton SL, Perkins AV, Cuffe JSM. The effect of gestational age on mitochondrial properties of the mouse placenta. *Reprod Fertil*. 2022;3(1):19-29. Epub 2022/03/17. doi: 10.1530/RAF-21-0064. PubMed PMID: 35291465; PMCID: PMC8897591.
27. Diaz M, Aragonés G, Sanchez-Infantes D, Bassols J, Perez-Cruz M, de Zegher F, Lopez-Bermejo A, Ibanez L. Mitochondrial DNA in placenta: associations with fetal growth and superoxide dismutase activity. *Horm Res Paediatr*. 2014;82(5):303-9. Epub 2014/09/24. doi: 10.1159/000366079. PubMed PMID: 25247554.
28. Lin XJ, Xu XX, Zhang HX, Ding MM, Cao WQ, Yu QY, Wang YH, Yan HT, Yang XJ. Placental mtDNA copy number and methylation in association with macrosomia in healthy pregnancy. *Placenta*. 2022;118:1-9. Epub 2022/01/01. doi: 10.1016/j.placenta.2021.12.021. PubMed PMID: 34972066.
29. Armstrong DA, Green BB, Blair BA, Guerin DJ, Litzky JF, Chavan NR, Pearson KJ, Marsit CJ. Maternal smoking during pregnancy is associated with mitochondrial DNA methylation. *Environ Epigenet*. 2016;2(3). Epub 2016/08/01. doi: 10.1093/eep/dvw020. PubMed PMID: 28979800; PMCID: PMC5624552.
30. Janssen BG, Byun HM, Gyselaers W, Lefebvre W, Baccarelli AA, Nawrot TS. Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. *Epigenetics*. 2015;10(6):536-44. Epub 2015/05/23. doi: 10.1080/15592294.2015.1048412. PubMed PMID: 25996590; PMCID: PMC4623402.
31. Vos S, Nawrot TS, Martens DS, Byun HM, Janssen BG. Mitochondrial DNA methylation in placental tissue: a proof of concept study by means of prenatal environmental stressors. *Epigenetics*. 2021;16(2):121-31. Epub 2020/07/14. doi: 10.1080/15592294.2020.1790923. PubMed PMID: 32657220; PMCID: PMC7889149.

32. Chen X, Zhou Y, Hu C, Xia W, Xu S, Cai Z, Li Y. Prenatal exposure to benzotriazoles and benzothiazoles and cord blood mitochondrial DNA copy number: A prospective investigation. *Environ Int.* 2020;143:105920. Epub 2020/07/13. doi: 10.1016/j.envint.2020.105920. PubMed PMID: 32653801.
  
33. Hu C, Sheng X, Li Y, Xia W, Zhang B, Chen X, Xing Y, Li X, Liu H, Sun X, Xu S. Effects of prenatal exposure to particulate air pollution on newborn mitochondrial DNA copy number. *Chemosphere.* 2020;253:126592. Epub 2020/04/15. doi: 10.1016/j.chemosphere.2020.126592. PubMed PMID: 32289600.
  
34. Smith AR, Lin PD, Rifas-Shiman SL, Rahman ML, Gold DR, Baccarelli AA, Claus Henn B, Amarasiwardena C, Wright RO, Coull B, Hivert MF, Oken E, Cardenas A. Prospective Associations of Early Pregnancy Metal Mixtures with Mitochondria DNA Copy Number and Telomere Length in Maternal and Cord Blood. *Environ Health Perspect.* 2021;129(11):117007. Epub 2021/11/20. doi: 10.1289/EHP9294. PubMed PMID: 34797165; PMCID: PMC8604047.
  
35. Gao D, Zhu B, Sun H, Wang X. Mitochondrial DNA Methylation and Related Disease. *Adv Exp Med Biol.* 2017;1038:117-32. Epub 2017/11/28. doi: 10.1007/978-981-10-6674-0\_9. PubMed PMID: 29178073.
  
36. Ryva BA, Zhou Z, Goodrich JM, Strakovsky RS. The potential role of environmental factors in modulating mitochondrial DNA epigenetic marks. *Vitam Horm.* 2023;122:107-45. Epub 2023/03/03. doi: 10.1016/bs.vh.2023.01.011. PubMed PMID: 36863791.
  
37. Zhou Z, Goodrich JM, Strakovsky RS. Mitochondrial Epigenetics and Environmental Health: Making a Case for Endocrine Disrupting Chemicals. *Toxicol Sci.* 2020;178(1):16-25. Epub 2020/08/11. doi: 10.1093/toxsci/kfaa129. PubMed PMID: 32777053; PMCID: PMC7818895.
  
38. Al-Qaraghoul M, Fang YMV. Effect of Fetal Sex on Maternal and Obstetric Outcomes. *Front Pediatr.* 2017;5:144. Epub 2017/07/05. doi: 10.3389/fped.2017.00144. PubMed PMID: 28674684; PMCID: PMC5476168.
  
39. de Zegher F, Devlieger H, Eeckels R. Fetal growth: boys before girls. *Horm Res.* 1999;51(5):258-9. Epub 1999/11/24. doi: 10.1159/000023382. PubMed PMID: 10559673.
  
40. Hirko KA, Comstock SS, Strakovsky RS, Kerver JM. Diet during Pregnancy and Gestational Weight Gain in a Michigan Pregnancy Cohort. *Curr Dev Nutr.* 2020;4(8):nzaa121. Epub 2020/08/15. doi: 10.1093/cdn/nzaa121. PubMed PMID: 32793851; PMCID: PMC7413979.

41. Kerver JM, Pearce EN, Ma T, Gentchev M, Elliott MR, Paneth N. Prevalence of inadequate and excessive iodine intake in a US pregnancy cohort. *Am J Obstet Gynecol*. 2021;224(1):82 e1-e8. Epub 2020/07/13. doi: 10.1016/j.ajog.2020.06.052. PubMed PMID: 32653458; PMCID: PMC7779669.
42. Slawinski BL, Talge N, Ingersoll B, Smith A, Glazier A, Kerver J, Paneth N, Racicot K. Maternal cytomegalovirus sero-positivity and autism symptoms in children. *Am J Reprod Immunol*. 2018;79(5):e12840. Epub 2018/03/10. doi: 10.1111/aji.12840. PubMed PMID: 29520885; PMCID: PMC5978736.
43. Haggerty DK, Strakovsky RS, Talge NM, Carignan CC, Glazier-Essalmi AN, Ingersoll BR, Karthikraj R, Kannan K, Paneth NS, Ruden DM. Prenatal phthalate exposures and autism spectrum disorder symptoms in low-risk children. *Neurotoxicol Teratol*. 2021;83:106947. Epub 2021/01/08. doi: 10.1016/j.ntt.2021.106947. PubMed PMID: 33412243; PMCID: PMC7825926.
44. Andreu AL, Martinez R, Marti R, Garcia-Arumi E. Quantification of mitochondrial DNA copy number: pre-analytical factors. *Mitochondrion*. 2009;9(4):242-6. Epub 2009/03/11. doi: 10.1016/j.mito.2009.02.006. PubMed PMID: 19272467.
45. Kupsco A, Bloomquist TR, Hu H, Reddam A, Tang D, Goldsmith J, Rundle AG, Baccarelli AA, Herbstman JB. Mitochondrial DNA copy number dynamics and associations with the prenatal environment from birth through adolescence in a population of Dominican and African American children. *Mitochondrion*. 2023;69:140-6. Epub 2023/02/23. doi: 10.1016/j.mito.2023.02.008. PubMed PMID: 36804466; PMCID: PMC10006332.
46. Wu M, Shu Y, Song L, Liu B, Zhang L, Wang L, Liu Y, Bi J, Xiong C, Cao Z, Xu S, Xia W, Li Y, Wang Y. Prenatal exposure to thallium is associated with decreased mitochondrial DNA copy number in newborns: Evidence from a birth cohort study. *Environ Int*. 2019;129:470-7. Epub 2019/06/04. doi: 10.1016/j.envint.2019.05.053. PubMed PMID: 31158593.
47. Novielli C, Mando C, Tabano S, Anelli GM, Fontana L, Antonazzo P, Miozzo M, Cetin I. Mitochondrial DNA content and methylation in fetal cord blood of pregnancies with placental insufficiency. *Placenta*. 2017;55:63-70. Epub 2017/06/19. doi: 10.1016/j.placenta.2017.05.008. PubMed PMID: 28623975.
48. Owa C, Poulin M, Yan L, Shioda T. Technical adequacy of bisulfite sequencing and pyrosequencing for detection of mitochondrial DNA methylation: Sources and avoidance of false-positive detection. *PLoS One*. 2018;13(2):e0192722. Epub 2018/02/09. doi: 10.1371/journal.pone.0192722. PubMed PMID: 29420656; PMCID: PMC5805350 not alter our adherence to PLOS ONE policies on sharing data and materials.

49. Basso O, Wilcox A. Mortality risk among preterm babies: immaturity versus underlying pathology. *Epidemiology*. 2010;21(4):521-7. Epub 2010/04/22. doi: 10.1097/EDE.0b013e3181debe5e. PubMed PMID: 20407380; PMCID: PMC2967434.
  
50. Talge NM, Mudd LM, Sikorskii A, Basso O. United States birth weight reference corrected for implausible gestational age estimates. *Pediatrics*. 2014;133(5):844-53. Epub 2014/04/30. doi: 10.1542/peds.2013-3285. PubMed PMID: 24777216.
  
51. Alexander GR, Himes JH, Kaufman RB, Mor J, Kogan M. A United States national reference for fetal growth. *Obstet Gynecol*. 1996;87(2):163-8. Epub 1996/02/01. doi: 10.1016/0029-7844(95)00386-X. PubMed PMID: 8559516.
  
52. American College of Obstetricians and Gynecologists. ACOG Committee Opinion No 579: Definition of term pregnancy. *Obstet Gynecol*. 2013;122(5):1139-40. Epub 2013/10/24. doi: 10.1097/01.AOG.0000437385.88715.4a. PubMed PMID: 24150030.
  
53. Priliani L, Prado EL, Restuadi R, Waturangi DE, Shankar AH, Malik SG. Maternal Multiple Micronutrient Supplementation Stabilizes Mitochondrial DNA Copy Number in Pregnant Women in Lombok, Indonesia. *J Nutr*. 2019;149(8):1309-16. Epub 2019/06/10. doi: 10.1093/jn/nxz064. PubMed PMID: 31177276; PMCID: PMC6686057.
  
54. Sanchez-Guerra M, Peng C, Trevisi L, Cardenas A, Wilson A, Osorio-Yanez C, Niedzwiecki MM, Zhong J, Svensson K, Acevedo MT, Solano-Gonzalez M, Amarasiriwardena CJ, Estrada-Gutierrez G, Brennan KJM, Schnaas L, Just AC, Laue HE, Wright RJ, Tellez-Royo MM, Wright RO, Baccarelli AA. Altered cord blood mitochondrial DNA content and pregnancy lead exposure in the PROGRESS cohort. *Environ Int*. 2019;125:437-44. Epub 2019/02/13. doi: 10.1016/j.envint.2019.01.077. PubMed PMID: 30753999; PMCID: PMC6391888.
  
55. Smith AR, Hinojosa Briseno A, Picard M, Cardenas A. The prenatal environment and its influence on maternal and child mitochondrial DNA copy number and methylation: A review of the literature. *Environ Res*. 2023;227:115798. Epub 2023/04/01. doi: 10.1016/j.envres.2023.115798. PubMed PMID: 37001851; PMCID: PMC10164709.
  
56. VanderWeele TJ, Robins JM. Directed acyclic graphs, sufficient causes, and the properties of conditioning on a common effect. *Am J Epidemiol*. 2007;166(9):1096-104. Epub 2007/08/19. doi: 10.1093/aje/kwm179. PubMed PMID: 17702973.
  
57. Duncan AF, Montoya-Williams D. Recommendations for Reporting Research About Racial Disparities in Medical and Scientific Journals. *JAMA Pediatr*. 2024. Epub 2024/01/02. doi: 10.1001/jamapediatrics.2023.5718. PubMed PMID: 38165691.

58. Green MS, Symons MJ. A comparison of the logistic risk function and the proportional hazards model in prospective epidemiologic studies. *J Chronic Dis.* 1983;36(10):715-23. Epub 1983/01/01. doi: 10.1016/0021-9681(83)90165-0. PubMed PMID: 6630407.
59. Boss J, Zhai J, Aung MT, Ferguson KK, Johns LE, McElrath TF, Meeker JD, Mukherjee B. Associations between mixtures of urinary phthalate metabolites with gestational age at delivery: a time to event analysis using summative phthalate risk scores. *Environ Health.* 2018;17(1):56. Epub 2018/06/22. doi: 10.1186/s12940-018-0400-3. PubMed PMID: 29925380; PMCID: PMC6011420.
60. Hu JMY, Arbuckle TE, Janssen P, Lanphear BP, Braun JM, Platt RW, Chen A, Fraser WD, McCandless LC. Associations of prenatal urinary phthalate exposure with preterm birth: the Maternal-Infant Research on Environmental Chemicals (MIREC) Study. *Can J Public Health.* 2020;111(3):333-41. Epub 2020/05/23. doi: 10.17269/s41997-020-00322-5. PubMed PMID: 32441020; PMCID: PMC7351891.
61. Crump C, Sundquist K, Winkleby MA, Sundquist J. Early-term birth (37-38 weeks) and mortality in young adulthood. *Epidemiology.* 2013;24(2):270-6. Epub 2013/01/23. doi: 10.1097/EDE.0b013e318280da0f. PubMed PMID: 23337240.
62. Reddy UM, Bettegowda VR, Dias T, Yamada-Kushnir T, Ko CW, Willinger M. Term pregnancy: a period of heterogeneous risk for infant mortality. *Obstet Gynecol.* 2011;117(6):1279-87. Epub 2011/05/25. doi: 10.1097/AOG.0b013e3182179e28. PubMed PMID: 21606738; PMCID: PMC5485902.
63. Beyerlein A. Quantile regression-opportunities and challenges from a user's perspective. *Am J Epidemiol.* 2014;180(3):330-1. Epub 2014/07/06. doi: 10.1093/aje/kwu178. PubMed PMID: 24989240.
64. Koenker R, Hallock KF. Quantile Regression. *Journal of Economic Perspectives.* 2001;15(4):143-56. doi: 10.1257/jep.15.4.143.
65. Koenker RW. Quantile regression: Cambridge University Press; 2005. 349 p.
66. Wasserstein RL, Lazar NA. The ASA statement on p-values: context, process, and purpose. Taylor & Francis; 2016. p. 129-33.
67. Amrhein V, Greenland S, McShane B. Scientists rise up against statistical significance. *Nature.* 2019;567(7748):305-7. Epub 2019/03/22. doi: 10.1038/d41586-019-00857-9. PubMed PMID: 30894741.

68. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology*. 1990;1(1):43-6. Epub 1990/01/01. PubMed PMID: 2081237.
69. Fukunaga H, Ikeda A. Mitochondrial DNA copy number variation across three generations: a possible biomarker for assessing perinatal outcomes. *Hum Genomics*. 2023;17(1):113. Epub 2023/12/15. doi: 10.1186/s40246-023-00567-4. PubMed PMID: 38098033; PMCID: PMC10722810.
70. Kuriyama S, Metoki H, Kikuya M, Obara T, Ishikuro M, Yamanaka C, Nagai M, Matsubara H, Kobayashi T, Sugawara J, Tamiya G, Hozawa A, Nakaya N, Tsuchiya N, Nakamura T, Narita A, Kogure M, Hirata T, Tsuji I, Nagami F, Fuse N, Arai T, Kawaguchi Y, Higuchi S, Sakaida M, Suzuki Y, Osumi N, Nakayama K, Ito K, Egawa S, Chida K, Kodama E, Kiyomoto H, Ishii T, Tsuboi A, Tomita H, Taki Y, Kawame H, Suzuki K, Ishii N, Ogishima S, Mizuno S, Takai-Igarashi T, Minegishi N, Yasuda J, Igarashi K, Shimizu R, Nagasaki M, Tanabe O, Koshiba S, Hashizume H, Motohashi H, Tominaga T, Ito S, Tanno K, Sakata K, Shimizu A, Hitomi J, Sasaki M, Kinoshita K, Tanaka H, Kobayashi T, Tohoku Medical Megabank Project Study G, Kure S, Yaegashi N, Yamamoto M. Cohort Profile: Tohoku Medical Megabank Project Birth and Three-Generation Cohort Study (TMM BirThree Cohort Study): rationale, progress and perspective. *Int J Epidemiol*. 2020;49(1):18-9m. Epub 2019/09/11. doi: 10.1093/ije/dyz169. PubMed PMID: 31504573; PMCID: PMC7124511.
71. Clemente DB, Casas M, Vilahur N, Begiristain H, Bustamante M, Carsin AE, Fernandez MF, Fierens F, Gyselaers W, Iniguez C, Janssen BG, Lefebvre W, Llop S, Olea N, Pedersen M, Pieters N, Santa Marina L, Souto A, Tardon A, Vanpoucke C, Vrijheid M, Sunyer J, Nawrot TS. Prenatal Ambient Air Pollution, Placental Mitochondrial DNA Content, and Birth Weight in the INMA (Spain) and ENVIRONAGE (Belgium) Birth Cohorts. *Environ Health Perspect*. 2016;124(5):659-65. Epub 2015/09/01. doi: 10.1289/ehp.1408981. PubMed PMID: 26317635; PMCID: PMC4858384 interests.
72. Colleoni F, Lattuada D, Garretto A, Massari M, Mando C, Somigliana E, Cetin I. Maternal blood mitochondrial DNA content during normal and intrauterine growth restricted (IUGR) pregnancy. *Am J Obstet Gynecol*. 2010;203(4):365 e1-6. Epub 2010/07/14. doi: 10.1016/j.ajog.2010.05.027. PubMed PMID: 20619387.
73. Lattuada D, Colleoni F, Martinelli A, Garretto A, Magni R, Radaelli T, Cetin I. Higher mitochondrial DNA content in human IUGR placenta. *Placenta*. 2008;29(12):1029-33. Epub 2008/11/15. doi: 10.1016/j.placenta.2008.09.012. PubMed PMID: 19007984.
74. Naha R, Anees A, Chakrabarty S, Naik PS, Pandove M, Pandey D, Satyamoorthy K. Placental mitochondrial DNA mutations and copy numbers in intrauterine growth restricted (IUGR) pregnancy. *Mitochondrion*. 2020;55:85-94. Epub 2020/08/31. doi: 10.1016/j.mito.2020.08.008. PubMed PMID: 32861875.

75. Gemma C, Sookoian S, Alvarinas J, Garcia SI, Quintana L, Kanevsky D, Gonzalez CD, Pirola CJ. Mitochondrial DNA depletion in small- and large-for-gestational-age newborns. *Obesity (Silver Spring)*. 2006;14(12):2193-9. Epub 2006/12/26. doi: 10.1038/oby.2006.257. PubMed PMID: 17189546.
76. Holland OJ, Hickey AJR, Alvsaker A, Moran S, Hedges C, Chamley LW, Perkins AV. Changes in mitochondrial respiration in the human placenta over gestation. *Placenta*. 2017;57:102-12. Epub 2017/09/03. doi: 10.1016/j.placenta.2017.06.011. PubMed PMID: 28863998.
77. Dwi Putra SE, Reichetzeder C, Hasan AA, Slowinski T, Chu C, Kramer BK, Kleuser B, Hoher B. Being Born Large for Gestational Age is Associated with Increased Global Placental DNA Methylation. *Sci Rep*. 2020;10(1):927. Epub 2020/01/24. doi: 10.1038/s41598-020-57725-0. PubMed PMID: 31969597; PMCID: PMC6976643.
78. Bartho LA, O'Callaghan JL, Fisher JJ, Cuffe JSM, Kaitu'u-Lino TJ, Hannan NJ, Clifton VL, Perkins AV. Analysis of mitochondrial regulatory transcripts in publicly available datasets with validation in placentae from pre-term, post-term and fetal growth restriction pregnancies. *Placenta*. 2021;112:162-71. Epub 2021/08/08. doi: 10.1016/j.placenta.2021.07.303. PubMed PMID: 34364121.
79. Northam S, Knapp TR. The reliability and validity of birth certificates. *J Obstet Gynecol Neonatal Nurs*. 2006;35(1):3-12. Epub 2006/02/10. doi: 10.1111/j.1552-6909.2006.00016.x. PubMed PMID: 16466348.
80. Ziogas C, Hillyer J, Saftlas AF, Spracklen CN. Validation of birth certificate and maternal recall of events in labor and delivery with medical records in the Iowa health in pregnancy study. *BMC Pregnancy Childbirth*. 2022;22(1):232. Epub 2022/03/24. doi: 10.1186/s12884-022-04581-7. PubMed PMID: 35317778; PMCID: PMC8939232.
81. Shao Z, Han Y, Zhou D. Optimized bisulfite sequencing analysis reveals the lack of 5-methylcytosine in mammalian mitochondrial DNA. *BMC Genomics*. 2023;24(1):439. Epub 2023/08/05. doi: 10.1186/s12864-023-09541-9. PubMed PMID: 37542258; PMCID: PMC10403921.



## APPENDIX A: TABLES

**Table 1.** Characteristics of women and infants in the full sample with mitochondria and birth outcome data, and the analytic sample with all covariate data.

Characteristic	Full Sample <sup>1</sup> (n=445)	Analytic Sample <sup>1</sup> (n=396)
	<b>Median (25<sup>th</sup>, 75<sup>th</sup> Percentiles)</b>	
Age, years*	25.3 (21.8, 29.5)	25.3 (21.8, 29.4)
Bloodspot collection, weeks*	11.0 (9.0, 14.0)	11.0 (9.0, 14.0)
hBG*	0.5 (0.4, 0.7)	0.6 (0.5, 0.7)
	<b>n (%)</b>	
Race/ethnicity*		
White (ref.)	260 (58.4)	230 (58.1)
Other <sup>2</sup>	182 (40.9)	166 (41.9)
Annual household Income*		
< \$25,000	286 (64.3)	269 (67.9)
≥ \$25,000 (ref.)	140 (31.5)	127 (32.1)
Education		
Did not finish high school	69 (15.5)	63 (15.9)
High school or GED	141 (31.7)	124 (31.3)
Some college	137 (30.8)	128 (32.3)
College graduate or more	87 (19.5)	74 (18.7)
Marital status		
Married, living with baby's father	121 (27.2)	105 (26.5)
Married	19 (4.3)	17 (4.3)
Unmarried, living with baby's father	162 (36.4)	144 (36.4)
Unmarried	142 (31.9)	130 (32.8)
Tobacco use*		
No (ref.)	318 (71.5)	282 (71.2)
Yes	126 (28.3)	114 (28.8)
Pre-pregnancy BMI, categories*		
Do not have obesity (ref.) <sup>3</sup>	312 (70.1)	279 (70.5)
Have obesity <sup>3</sup>	133 (29.9)	117 (29.5)
Parity*		
0 children (ref.)	181 (40.7)	160 (40.4)
1+ children	264 (59.3)	236 (59.6)
Basis of gestational age estimate		
Last menstrual period	354 (79.5)	307 (77.5)
Obstetric	91 (20.5)	89 (22.5)
Fetal sex*		
Female (ref.)	223 (50.1)	202 (51.0)
Male	222 (49.9)	194 (49.0)
Size for gestational age		
Small	45 (10.1)	38 (9.6)
Appropriate	358 (80.5)	320 (80.8)
Large	42 (9.4)	38 (9.6)

An \* indicates covariates included in the final model.

<sup>1</sup>Not all % equal 100. Missing from the full sample: age (n=9), gestational week of bloodspot collection (n=20), race/ethnicity (n=3), annual household income (n=19), education (n=11), marital status, tobacco use (n=1). Missing from the analytic sample: education (n=7).

<sup>2</sup>Other refers to Black or African American, American Indian, Alaska Native, Asian, Hawaiian/Pacific Islander or Hispanic. <sup>3</sup>Do not have obesity: BMI < 30 kg/m<sup>2</sup>; have obesity: BMI ≥ 30 kg/m<sup>2</sup>.

BMI, body mass index; hBG, human β-globin.

**Table 2.** Distribution of the mitochondrial DNA biomarkers in the analytic sample (n=396).

Gene and site	n	% > 0	Overall	Females	Males
			Median (25 <sup>th</sup> , 75 <sup>th</sup> Percentiles)		
<i>ND1</i>	396	100.0	0.6 (0.5, 0.7)	0.6 (0.5, 0.7)	0.6 (0.4, 0.7)
<i>LDLR</i> CpG1	351	86.5	2.8 (2.1, 3.6)	2.8 (1.9, 3.5)	2.8 (2.2, 3.7)
<i>LDLR</i> CpG2	316	18.5	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
<i>LDLR</i> CpG3	196	47.4	0.0 (0.0, 2.2)	0.0 (0.0, 2.3)	1.0 (0.0, 2.2)
<i>LDLR</i> Average	351	86.8	1.4 (0.9, 2.2)	1.3 (0.8, 2.0)	1.5 (1.0, 2.3)
<i>DLOOP</i> CpG1	347	60.3	0.7 (0.0, 1.1)	0.7 (0.0, 1.1)	0.7 (0.0, 1.1)
<i>DLOOP</i> CpG2	304	40.3	0.0 (0.0, 0.9)	0.0 (0.0, 0.9)	0.0 (0.0, 0.8)
<i>DLOOP</i> CpG3	286	31.8	0.0 (0.0, 0.7)	0.0 (0.0, 0.7)	0.0 (0.0, 0.7)
<i>DLOOP</i> Average	347	61.5	0.3 (0.0, 0.9)	0.4 (0.0, 0.9)	0.4 (0.0, 0.9)
<i>CYTB</i> CpG1	306	32.6	0.0 (0.0, 1.8)	0.0 (0.0, 1.7)	0.0 (0.0, 1.8)
<i>CYTB</i> CpG2	295	36.5	0.0 (0.0, 2.6)	0.0 (0.0, 2.4)	0.0 (0.0, 2.7)
<i>CYTB</i> CpG3	291	18.67	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
<i>CYTB</i> Average	306	49.0	0.0 (0.0, 1.2)	0.0 (0.0, 1.2)	0.4 (0.0, 1.2)
<i>MTTF</i> S1 CpG1	308	30.7	0.0 (0.0, 0.9)	0.0 (0.0, 1.0)	0.0 (0.0, 0.9)
<i>MTTF</i> S2 CpG1	298	89.2	4.2 (2.6, 5.1)	4.2 (2.7, 5.0)	4.2 (2.6, 5.2)
<i>MTTF</i> S2 CpG2	260	81.9	5.1 (3.6, 7.4)	5.5 (3.8, 7.5)	4.9 (3.5, 7.3)
<i>MTTF</i> S2 Average	298	93.2	4.7 (3.0, 5.7)	4.8 (2.9, 5.7)	4.6 (3.1, 5.6)
<i>CYTB</i> , cytochrome b; <i>DLOOP</i> , D-loop promoter region, heavy strand; <i>LDLR</i> , D-loop promoter region, light strand; mtDNA, mitochondrial DNA; <i>MTTF</i> , mitochondrially encoded tRNA phenylalanine; <i>ND1</i> , NADH-ubiquinone oxidoreductase chain 1; S1, location 1; S2, location 2.					

**Table 3.** Distribution of the birth outcomes in the ARCH analytic sample (n=396).

	Overall	Female	Male
Outcome	Median (25 <sup>th</sup> , 75 <sup>th</sup> Percentiles)		
Birthweight, grams	3345 (3005, 3643)	3343 (3005, 3629)	3345 (3005, 3646)
Birthweight z-score <sup>1</sup>	-0.1 (-0.7, 0.6)	0.0 (-0.6, 0.7)	-0.2 (-0.8, 0.5)
Gestational length, weeks <sup>2</sup>	39.4 (38.4, 40.4)	39.4 (38.7, 40.6)	39.3 (38.3, 40.1)
Gestational length, days <sup>3</sup>	276.0 (269.0, 282.5)	276.0 (271.0, 284.0)	275.0 (268.0, 281.0)
Gestational length, < 37 weeks	n (%)		
Yes	33 (8.3)	17 (8.4)	16 (8.3)
No	363 (91.7)	185 (91.6)	178 (91.7)
Gestational length, < 39 weeks			
Yes	125 (31.6)	58 (28.7)	67 (34.5)
No	271 (68.4)	144 (71.3)	127 (65.5)
<sup>1</sup> Birthweight for gestational age z-score was calculated using a U.S. population-based reference (50).			
<sup>2</sup> Gestation length was reported as the last menstrual period-based or obstetric-based estimate as determined using the algorithm by Basso and Wilcox (49).			

**Table 4.** Associations between maternal mitochondrial DNA methylation and birthweight, overall and by fetal sex, using linear regression.

Gene/site	Birthweight			<i>p-int</i>
	Overall n= 196-351	Females n= 101-178	Males n=95-173	
	$\beta$ (95% CI)			
<i>LDLR</i> CpG1 <sup>1</sup>	26.2 (-54.0, 106.5)	45.7 (-71.0, 162.3)	10.7 (-94.4, 115.7)	0.7
<i>LDLR</i> CpG2 <sup>1</sup>	30.2 (-73.8, 134.1)	76.6 (-55.7, 208.9)	-34.6 (-189, 119.8)	0.3
<i>LDLR</i> CpG3 <sup>1</sup>	55.4 (-33.0, 143.8)	93.7 (-33.0, 220.5)	22.6 (-95.2, 140.3)	0.4
<i>LDLR</i> Average <sup>1</sup>	43.7 (-55.5, 142.8)	76.0 (-58.4, 210.4)	11.6 (-122.2, 145.5)	0.5
<i>DLOOP</i> CpG1 <sup>1</sup>	23.0 (-58.6, 104.6)	34.4 (-73.9, 142.6)	8.2 (-114.8, 131.2)	0.6
<i>DLOOP</i> CpG2 <sup>1</sup>	30.9 (-81.0, 142.7)	16.6 (-137.5, 170.6)	46.5 (-114.5, 207.5)	0.8
<i>DLOOP</i> CpG3 <sup>1</sup>	11.4 (-99.6, 122.4)	-25.5 (-171.4, 120.4)	58.3 (-105.3, 221.9)	0.4
<i>DLOOP</i> Average <sup>1</sup>	42.4 (-62.6, 147.4)	29.6 (-112.6, 171.8)	57.2 (-95.0, 209.4)	0.8
<i>CYTB</i> CpG1 <sup>1</sup>	14.8 (-53.8, 83.4)	47.4 (-49.3, 144.2)	-14.7 (-107.0, 77.6)	0.4
<i>CYTB</i> CpG2 <sup>1</sup>	21.3 (-36.3, 78.8)	41.7 (-36.2, 119.6)	-3.1 (-88.2, 82.0)	0.4
<i>CYTB</i> CpG3 <sup>1</sup>	<b>66.5 (-9.7, 142.6)<sup>#</sup></b>	31.2 (-73.6, 136.0)	<b>105.3 (-4.6, 215.2)<sup>#</sup></b>	0.3
<i>CYTB</i> Average <sup>1</sup>	33.3 (-45.2, 111.9)	43.7 (-67.0, 154.4)	23.2 (-86.3, 132.7)	0.8
<i>MTTF</i> S1 CpG1 <sup>1</sup>	<b>67.9 (-17.9, 153.6)<sup>#</sup></b>	-12.1 (-135.5, 111.3)	<b>143.2 (23.5, 262.9)<sup>*</sup></b>	<b>0.1</b>
<i>MTTF</i> S2 CpG1 <sup>2</sup>	16.6 (-18.5, 51.7)	16.8 (-29.7, 63.4)	16.3 (-32.5, 65.2)	1.0
<i>MTTF</i> S2 CpG2 <sup>2</sup>	10.9 (-24.4, 46.3)	9.8 (-31.1, 50.6)	12.5 (-32.6, 57.6)	0.9
<i>MTTF</i> S2 Average <sup>2</sup>	10.4 (-24.6, 45.3)	6.2 (-38.4, 50.9)	15.0 (-31.6, 61.5)	0.8

All models accounted for plate number, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. **Bolded values are considered meaningful.** \* $p \leq 0.05$ ; <sup>#</sup> $p < 0.10$ .

<sup>1</sup>Gene sites that are ln-transformed and  $\beta$ -estimates (95% CI) were back-transformed using the following equation:  $\beta * \ln(2)$ . Results interpreted as the change in birthweight (g) for each two-fold increase in mtDNA methylation.

<sup>2</sup>Gene sites not transformed and  $\beta$ -estimates (95% CI) were not transformed. Results interpreted as the change in birthweight Z-score for each one-unit increase in mtDNA methylation.

BMI, body mass index; *CYTB*, cytochrome b; *DLOOP*, D-loop promoter region, heavy strand; *hBG*, human  $\beta$ -globin; *LDLR*, D-loop promoter region, light strand; mtDNA, mitochondrial DNA; *MTTF*, mitochondrially encoded tRNA phenylalanine; S1, location 1; S2, location 2.

**Table 5.** Associations between maternal mitochondrial DNA methylation and birthweight z-score, overall and by fetal sex, using linear regression.

Gene/site	Birthweight z-score			<i>p-int</i>
	Overall n= 196-351	Females n= 101-178	Males n=95-173	
	$\beta$ (95% CI)			
<i>LDLR</i> CpG1 <sup>1</sup>	0.07 (-0.08, 0.21)	0.10 (-0.11, 0.31)	0.04 (-0.15, 0.23)	0.7
<i>LDLR</i> CpG2 <sup>1</sup>	<b>0.15 (-0.04, 0.33)<sup>#</sup></b>	<b>0.22 (-0.02, 0.46)<sup>#</sup></b>	0.05 (-0.23, 0.33)	0.3
<i>LDLR</i> CpG3 <sup>1</sup>	<b>0.14 (-0.01, 0.30)<sup>#</sup></b>	<b>0.19 (-0.03, 0.42)<sup>#</sup></b>	0.10 (-0.11, 0.31)	0.5
<i>LDLR</i> Average <sup>1</sup>	0.11 (-0.07, 0.29)	0.16 (-0.08, 0.40)	0.05 (-0.19, 0.30)	0.5
<i>DLOOP</i> CpG1 <sup>1</sup>	-0.01 (-0.16, 0.14)	0.07 (-0.13, 0.27)	-0.13 (-0.35, 0.10)	<b>0.2</b>
<i>DLOOP</i> CpG2 <sup>1</sup>	0.00 (-0.21, 0.21)	-0.04 (-0.33, 0.24)	0.05 (-0.26, 0.35)	0.7
<i>DLOOP</i> CpG3 <sup>1</sup>	0.00 (-0.22, 0.22)	-0.08 (-0.37, 0.20)	0.11 (-0.21, 0.43)	0.3
<i>DLOOP</i> Average <sup>1</sup>	0.01 (-0.18, 0.21)	0.04 (-0.22, 0.31)	-0.02 (-0.30, 0.26)	0.7
<i>CYTB</i> CpG1 <sup>1</sup>	0.00 (-0.12, 0.12)	0.01 (-0.16, 0.18)	-0.01 (-0.17, 0.16)	0.9
<i>CYTB</i> CpG2 <sup>1</sup>	0.02 (-0.08, 0.12)	0.08 (-0.06, 0.22)	-0.05 (-0.20, 0.10)	0.2
<i>CYTB</i> CpG3 <sup>1</sup>	<b>0.11 (-0.03, 0.24)<sup>#</sup></b>	0.06 (-0.13, 0.25)	<b>0.16 (-0.03, 0.36)<sup>#</sup></b>	0.5
<i>CYTB</i> Average <sup>1</sup>	0.05 (-0.09, 0.19)	0.08 (-0.12, 0.28)	0.02 (-0.17, 0.22)	0.7
<i>MTTF</i> S1 CpG1 <sup>1</sup>	0.06 (-0.09, 0.22)	-0.05 (-0.27, 0.17)	<b>0.17 (-0.04, 0.38)<sup>#</sup></b>	<b>0.2</b>
<i>MTTF</i> S2 CpG1 <sup>2</sup>	0.04 (-0.02, 0.10)	0.03 (-0.05, 0.11)	0.06 (-0.03, 0.14)	0.7
<i>MTTF</i> S2 CpG2 <sup>2</sup>	0.04 (-0.03, 0.10)	0.03 (-0.05, 0.10)	0.05 (-0.03, 0.13)	0.6
<i>MTTF</i> S2 Average <sup>2</sup>	<b>0.05 (-0.01, 0.11)<sup>#</sup></b>	0.03 (-0.05, 0.11)	<b>0.07 (-0.01, 0.15)<sup>#</sup></b>	0.5
All models accounted for plate number, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. <b>Bolded values are considered meaningful.</b> * <i>p</i> ≤0.05; <sup>#</sup> <i>p</i> ≤0.10.				
<sup>1</sup> Gene sites that are ln-transformed and $\beta$ -estimates (95% CI) were back-transformed using the following equation: $\beta \cdot \ln(2)$ . Results interpreted as the change in birthweight z-score for each two-fold increase in mtDNA methylation.				
<sup>2</sup> Gene sites not transformed and $\beta$ -estimates (95% CI) were not transformed. Results interpreted as the change in birthweight Z-score for each one-unit increase in mtDNA methylation.				
BMI, body mass index; <i>CYTB</i> , cytochrome b; <i>DLOOP</i> , D-loop promoter region, heavy strand; <i>hBG</i> , human $\beta$ -globin; <i>LDLR</i> , D-loop promoter region, light strand; mtDNA, mitochondrial DNA; <i>MTTF</i> , mitochondrially encoded tRNA phenylalanine S1, location 1; S2, location 2.				

**Table 6.** Associations between maternal mitochondrial DNA methylation with gestation length, overall and by fetal sex, using Cox Proportional Hazards models.

Gene/site	Gestational length			<i>p-int</i> <sup>4</sup>
	Overall n= 196-351	Females <sup>3</sup> n= 101-178	Males <sup>3</sup> n= 95-173	
	HR (95% CI)			
<i>LDLR</i> CpG1 <sup>1</sup>	1.1 (0.8, 1.4)	1.3 (0.9, 2.0)	0.9 (0.6, 1.4)	<b>0.2</b>
<i>LDLR</i> CpG2 <sup>1</sup>	1.1 (0.8, 1.5)	1.3 (0.8, 2.0)	1.1 (0.7, 1.6)	0.4
<i>LDLR</i> CpG3 <sup>1</sup>	1.0 (0.8, 1.3)	1.1 (0.8, 1.7)	0.9 (0.6, 1.5)	0.6
<i>LDLR</i> Average <sup>1</sup>	1.1 (0.8, 1.5)	1.4 (0.8, 2.3)	0.9 (0.5, 1.4)	<b>0.1</b>
<i>DLOOP</i> CpG1 <sup>1</sup>	0.8 (0.6, 1.1)	<b>0.7 (0.4, 1.1)<sup>#</sup></b>	1.0 (0.7, 1.4)	0.3
<i>DLOOP</i> CpG2 <sup>1</sup>	1.0 (0.7, 1.4)	1.0 (0.6, 1.8)	0.9 (0.5, 1.5)	0.6
<i>DLOOP</i> CpG3 <sup>1</sup>	1.0 (0.7, 1.5)	0.9 (0.5, 1.8)	1.0 (0.6, 1.7)	1.0
<i>DLOOP</i> Average <sup>1</sup>	0.9 (0.6, 1.3)	0.7 (0.4, 1.3)	1.0 (0.6, 1.6)	0.5
<i>CYTB</i> CpG1 <sup>1</sup>	1.0 (0.8, 1.3)	1.3 (0.9, 1.7)	0.8 (0.6, 1.1)	<b>0.1</b>
<i>CYTB</i> CpG2 <sup>1</sup>	1.1 (0.9, 1.3)	<b>1.3 (1.0, 1.7)<sup>#</sup></b>	1.0 (0.8, 1.3)	<b>0.2</b>
<i>CYTB</i> CpG3 <sup>1</sup>	1.0 (0.8, 1.3)	1.0 (0.7, 1.5)	1.0 (0.7, 1.4)	1.0
<i>CYTB</i> Average <sup>1</sup>	1.1 (0.8, 1.4)	1.3 (0.9, 1.9)	0.9 (0.6, 1.4)	0.3
<i>MTTF</i> S1 CpG1 <sup>1</sup>	0.8 (0.6, 1.1)	0.8 (0.5, 1.2)	0.8 (0.5, 1.3)	0.8
<i>MTTF</i> S2 CpG1 <sup>2</sup>	1.0 (0.9, 1.1)	1.1 (0.9, 1.3)	1.0 (0.8, 1.1)	0.3
<i>MTTF</i> S2 CpG2 <sup>2</sup>	1.0 (0.9, 1.2)	1.0 (0.8, 1.2)	1.1 (0.9, 1.2)	0.4
<i>MTTF</i> S2 Average <sup>2</sup>	1.1 (0.9, 1.2)	1.1 (0.9, 1.4)	1.0 (0.9, 1.2)	0.2

All Cox PHMs accounted for plate number, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. **Bolded values are considered meaningful.** \* $p \leq 0.05$ ; <sup>#</sup> $p \leq 0.10$ .

<sup>1</sup>Gene sites are ln-transformed and HRs (95% CIs) were back-transformed using the following equation:  $e^{\log(\text{HR}) * \log(2)}$ . Results interpreted as the percent change in the HR for each two-fold increase in mtDNA methylation.

<sup>2</sup>Gene sites not transformed and HRs (95% CIs) were not adjusted. Results interpreted as the HR for each one-unit increase in mtDNA methylation.

<sup>3</sup>p-values calculated from PHMs including either only females or only males.

<sup>4</sup>p-int was derived from a model with an interaction p-value.

BMI, body mass index; *CYTB*, cytochrome b; *DLOOP*, D-loop promoter region, heavy strand; *hBG*, human  $\beta$ -globin; HR, hazard ratio; *LDLR*, D-loop promoter region, light strand; mtDNA, mitochondrial DNA; *MTTF*, mitochondrially encoded tRNA phenylalanine; PHM, proportional hazards model; S1, location 1; S2, location 2.

**Table 7.** Associations between maternal mtDNAcn and mtDNA methylation with gestational length overall, using quantile regression.

Quantile	5 <sup>th</sup> Percentile	25 <sup>th</sup> Percentile	50 <sup>th</sup> Percentile	75 <sup>th</sup> Percentile	95 <sup>th</sup> Percentile
Corresponding Gestational Length	36.0 weeks	38.4 weeks	39.4 weeks	40.4 weeks	41.4 weeks
Gene/site	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)
<i>NDI</i> <sup>1</sup>	6.5 (-1.8, 14.7)	1.9 (-1.9, 5.7)	0.1 (-2.8, 2.9)	0.9 (-1.1, 2.9)	1.7 (-1.7, 5.0)
<i>LDLR</i> CpG1 <sup>1</sup>	0.5 (-4.6, 5.7)	-1.0 (-3.5, 1.5)	0.4 (-1.4, 2.2)	-0.1 (-1.7, 1.4)	-0.2 (-2.1, 1.8)
<i>LDLR</i> CpG2 <sup>1</sup>	0.6 (-6.2, 7.3)	-1.2 (-4.8, 2.4)	-0.6 (-2.8, 1.6)	0.1 (-1.9, 2.0)	<b>1.9 (-0.1, 3.9) #</b>
<i>LDLR</i> CpG3 <sup>1</sup>	-2.8 (-8.0, 2.4)	0.3 (-2.2, 2.8)	0.6 (-1.6, 2.7)	-0.4 (-1.8, 1.0)	<b>2.7 (0.9, 4.4) *</b>
<i>LDLR</i> Average <sup>1</sup>	1.1 (-6.0, 8.2)	-1.2 (-4.3, 2.0)	0.4 (-1.8, 2.6)	-0.1 (-2.0, 1.8)	0.2 (-2.1, 2.6)
<i>DLOOP</i> CpG1 <sup>1</sup>	2.9 (-2.0, 7.8)	1.6 (-1.0, 4.1)	0.7 (-1.1, 2.6)	0.2 (-1.4, 1.9)	-0.9 (-2.5, 0.6)
<i>DLOOP</i> CpG2 <sup>1</sup>	5.1 (-2.9, 13.1)	1.2 (-2.1, 4.5)	0.9 (-1.5, 3.2)	1.1 (-0.8, 3.0)	<b>-2.7 (-5.6, 0.2) #</b>
<i>DLOOP</i> CpG3 <sup>1</sup>	-2.7 (-11.8, 6.5)	-0.3 (-3.7, 3.2)	1.3 (-1.5, 4.1)	0.3 (-1.7, 2.3)	0.1 (-2.6, 2.7)
<i>DLOOP</i> Average <sup>1</sup>	4.8 (-1.1, 10.7)	1.2 (-2.0, 4.5)	1.1 (-1.3, 3.4)	0.8 (-1.2, 2.9)	<b>-1.9 (-3.8, 0.0) *</b>
<i>CYTB</i> CpG1 <sup>1</sup>	<b>4.5 (1.7, 7.3) *</b>	0.3 (-1.9, 2.5)	0.9 (-0.6, 2.4)	<b>0.9 (-0.1, 1.9) #</b>	0.9 (-0.8, 2.6)
<i>CYTB</i> CpG2 <sup>1</sup>	1.2 (-1.1, 3.5)	-0.3 (-2.2, 1.5)	-0.5 (-1.7, 0.7)	0.6 (-0.2, 1.4)	-0.4 (-1.3, 0.6)
<i>CYTB</i> CpG3 <sup>1</sup>	<b>2.3 (-0.2, 4.7) #</b>	0.2 (-2.2, 2.6)	0.3 (-1.4, 2.0)	<b>1.0 (-0.2, 2.2) #</b>	0.8 (-0.2, 1.7)
<i>CYTB</i> Average <sup>1</sup>	2.2 (-0.8, 5.3)	-0.3 (-2.8, 2.1)	0.4 (-1.4, 2.2)	<b>0.9 (-0.2, 2.0) #</b>	1.2 (-0.5, 3.0)
<i>MTTF</i> S1 CpG1 <sup>1</sup>	<b>8.2 (3.9, 12.5) *</b>	1.9 (-0.7, 4.6)	1.0 (-1.0, 3.0)	-0.4 (-1.9, 1.2)	-0.8 (-2.6, 1.0)
<i>MTTF</i> S2 CpG1 <sup>2</sup>	-0.7 (-3.1, 1.6)	-0.3 (-1.6, 1.0)	-0.1 (-1.0, 0.7)	0.1 (-0.4, 0.7)	0.4 (-0.5, 1.3)
<i>MTTF</i> S2 CpG2 <sup>2</sup>	0.4 (-1.4, 2.1)	-0.6 (-1.8, 0.6)	-0.1 (-0.9, 0.7)	-0.3 (-0.9, 0.2)	<b>0.7 (-0.1, 1.5) #</b>
<i>MTTF</i> S2 Average <sup>2</sup>	-1.1 (-2.9, 0.6)	-0.7 (-2.0, 0.5)	-0.4 (-1.1, 0.4)	0.1 (-0.5, 0.6)	<b>0.7 (0.0, 1.5) *</b>

Covariate-adjusted quantile regression models accounted for plate number or hBG, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. **Bolded values are considered meaningful.** \* $p \leq 0.05$ ; # $p \leq 0.10$ .

<sup>1</sup>Gene sites were ln-transformed and  $\beta$  (95% CIs) were back-transformed using the following equation:  $\beta * \ln(2)$ . Results interpreted as the percent change in gestation length for each two-fold increase in mtDNAcn or mtDNA methylation.

<sup>2</sup>Gene sites not transformed and  $\beta$  (95% CIs) were not adjusted. Results interpreted as the change in gestation length (in days) for each one-unit increase in mtDNA methylation.

BMI, body mass index; *CYTB*, cytochrome b; *DLOOP*, D-loop promoter region, heavy strand; *hBG*, human  $\beta$ -globin; *LDLR*, D-loop promoter region, light strand; *MTTF*, mitochondrially encoded tRNA phenylalanine; *NDI*, NADH-ubiquinone oxidoreductase chain 1; S1, location 1; S2, location 2.

**Table 8.** Associations between maternal mtDNA and mtDNA methylation with gestational length in females, using quantile regression.

Quantile	5 <sup>th</sup> Percentile	25 <sup>th</sup> Percentile	50 <sup>th</sup> Percentile	75 <sup>th</sup> Percentile	95 <sup>th</sup> Percentile
Corresponding Gestational Length	36.1 weeks	38.7 weeks	39.4 weeks	40.6 weeks	41.7 weeks
Gene/site	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)
<i>NDI</i> <sup>1</sup>	8.0 (-6.0, 22.1)	0.6 (-4.6, 5.8)	-2.3 (-5.4, 0.9)	-1.0 (-5.0, 3.0)	<b>-3.1 (-6.6, 0.4)</b> #
<i>LDLR</i> CpG1 <sup>1</sup>	2.0 (-2.0, 5.9)	-0.9 (-4.4, 2.6)	-1.1 (-3.5, 1.3)	0.1 (-2.2, 2.4)	1.7 (-0.5, 3.9)
<i>LDLR</i> CpG2 <sup>1</sup>	1.8 (-2.6, 6.3)	<b>-4.8 (-8.7, -1.0)</b> *	-0.9 (-4.0, 2.2)	-1.6 (-4.9, 1.7)	<b>3.3 (1.4, 5.1)</b> *
<i>LDLR</i> CpG3 <sup>1</sup>	1.6 (-33.0, 36.2)	0.4 (-3.4, 4.2)	0.9 (-1.7, 3.6)	-0.8 (-3.2, 1.7)	<b>1.8 (0.1, 3.4)</b> *
<i>LDLR</i> Average <sup>1</sup>	2.6 (-3.5, 8.8)	-2.0 (-6.3, 2.4)	-0.5 (-3.3, 2.3)	-1.7 (-4.6, 1.2)	<b>3.0 (1.0, 4.9)</b> *
<i>DLOOP</i> CpG1 <sup>1</sup>	3.6 (-7.4, 14.6)	<b>3.9 (0.8, 6.9)</b> *	<b>2.9 (0.2, 5.5)</b> *	<b>3.4 (1.1, 5.6)</b> *	1.9 (-1.0, 4.7)
<i>DLOOP</i> CpG2 <sup>1</sup>	-5.8 (-30.4, 18.7)	0.4 (-4.3, 5.0)	1.6 (-1.5, 4.7)	0.8 (-2.9, 4.4)	<b>3.0 (-0.6, 6.6)</b> #
<i>DLOOP</i> CpG3 <sup>1</sup>	-0.1 (-6.6, 6.5)	2.0 (-2.0, 6.0)	1.6 (-2.4, 5.5)	-0.5 (-3.9, 2.9)	0.1 (-4.1, 4.2)
<i>DLOOP</i> Average <sup>1</sup>	4.1 (-8.1, 16.4)	<b>4.1 (0.2, 8.1)</b> *	<b>2.7 (-0.5, 5.8)</b> #	<b>4.4 (0.7, 8.2)</b> *	1.8 (-2.3, 5.9)
<i>CYTB</i> CpG1 <sup>1</sup>	-1.1 (-12.1, 9.9)	0.0 (-2.6, 2.6)	1.1 (-0.8, 3.0)	0.6 (-1.2, 2.4)	1.0 (-2.0, 4.0)
<i>CYTB</i> CpG2 <sup>1</sup>	3.0 (-7.2, 13.2)	-0.8 (-2.9, 1.2)	-0.8 (-2.5, 0.9)	0.4 (-1.4, 2.2)	<b>0.8 (0.1, 1.4)</b> *
<i>CYTB</i> CpG3 <sup>1</sup>	2.2 (-10.5, 14.9)	-0.8 (-3.4, 1.8)	<b>2.8 (0.3, 5.4)</b> *	<b>2.3 (0.1, 4.5)</b> *	1.6 (-0.5, 3.7)
<i>CYTB</i> Average <sup>1</sup>	3.0 (-16.1, 22.0)	-0.5 (-3.3, 2.2)	<b>2.0 (0.0, 4.0)</b> *	1.2 (-0.9, 3.4)	<b>3.2 (0.5, 5.9)</b>
<i>MTTF</i> S1 CpG1 <sup>1</sup>	<b>10.4 (4.8, 16.0)</b> *	2.6 (-0.8, 6.0)	<b>3.1 (0.4, 5.8)</b> *	1.1 (-1.1, 3.2)	0.0 (-3.4, 3.3)
<i>MTTF</i> S2 CpG1 <sup>2</sup>	-0.1 (-3.7, 3.6)	-0.4 (-2.0, 1.2)	-0.2 (-1.3, 0.9)	0.0 (-1.2, 1.1)	<b>1.1 (1.0, 1.3)</b> *
<i>MTTF</i> S2 CpG2 <sup>2</sup>	0.4 (-11.7, 12.5)	-0.4 (-1.7, 0.9)	0.2 (-1.0, 1.4)	-0.3 (-1.2, 0.6)	<b>0.8 (0.5, 1.0)</b> *
<i>MTTF</i> S2 Average <sup>2</sup>	0.5 (-3.2, 4.1)	-0.7 (-2.2, 0.7)	-0.4 (-1.5, 0.7)	0.0 (-1.2, 1.1)	<b>0.9 (0.1, 1.7)</b> *

Covariate-adjusted quantile regression models accounted for plate number or hBG, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. **Bolded values are considered meaningful.** \* $p \leq 0.05$ ; # $p \leq 0.10$ .

<sup>1</sup>Gene sites were ln-transformed and  $\beta$  (95% CIs) were back-transformed using the following equation:  $\beta * \ln(2)$ . Results interpreted as the percent change in gestation length for each two-fold increase in mtDNAcn or mtDNA methylation.

<sup>2</sup>Gene sites not transformed and  $\beta$  (95% CIs) were not adjusted. Results interpreted as the change in gestation length (in days) for each one-unit increase in mtDNA methylation.

BMI, body mass index; *CYTB*, cytochrome b; *DLOOP*, D-loop promoter region, heavy strand; *hBG*, human  $\beta$ -globin; *LDLR*, D-loop promoter region, light strand; *MTTF*, mitochondrially encoded tRNA phenylalanine; *NDI*, NADH-ubiquinone oxidoreductase chain 1; S1, location 1; S2, location 2.



**Table 9.** Associations between maternal mtDNA and mtDNA methylation with gestation length in males, using quantile regression.

Quantile	5 <sup>th</sup> Percentile	25 <sup>th</sup> Percentile	50 <sup>th</sup> Percentile	75 <sup>th</sup> Percentile	95 <sup>th</sup> Percentile
Corresponding Gestational Length	36.0 weeks	38.3 weeks	39.3 weeks	40.1 weeks	41.3 weeks
Gene/site	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)	$\beta$ (95%CI)
<i>NDI</i> <sup>1</sup>	<b>3.3 (0.2, 6.5) *</b>	3.8 (-2.8, 10.4)	0.0 (-4.0, 4.0)	0.7 (-2.7, 4.1)	2.7 (-4.2, 9.7)
<i>LDLR</i> CpG1 <sup>1</sup>	-1.6 (-6.6, 3.4)	0.1 (-4.5, 4.7)	0.5 (-2.5, 3.5)	0.7 (-1.8, 3.2)	-0.9 (-5.0, 3.3)
<i>LDLR</i> CpG2 <sup>1</sup>	<b>-3.3 (-6.3, -0.3) *</b>	0.5 (-5.0, 6.0)	-0.5 (-3.5, 2.5)	1.0 (-1.6, 3.7)	-2.7 (-8.6, 3.1)
<i>LDLR</i> CpG3 <sup>1</sup>	<b>2.6 (0.4, 4.8) *</b>	0.2 (-4.4, 4.8)	-0.8 (-4.0, 2.4)	-0.9 (-3.5, 1.7)	0.3 (-5.1, 5.6)
<i>LDLR</i> Average <sup>1</sup>	-2.0 (-6.8, 2.8)	1.6 (-3.7, 6.9)	0.0 (-3.7, 3.7)	0.7 (-2.0, 3.5)	-2.0 (-6.7, 2.7)
<i>DLOOP</i> CpG1 <sup>1</sup>	<b>6.6 (3.3, 10.0) *</b>	0.7 (-3.7, 5.0)	0.3 (-2.2, 2.7)	<b>-1.7 (-3.7, 0.2) #</b>	<b>-2.8 (-5.8, 0.1) #</b>
<i>DLOOP</i> CpG2 <sup>1</sup>	-1.4 (-8.5, 5.7)	3.6 (-1.9, 9.1)	1.5 (-2.2, 5.2)	<b>2.8 (0.1, 5.6) *</b>	-2.8 (-8.2, 2.7)
<i>DLOOP</i> CpG3 <sup>1</sup>	-1.5 (-8.6, 5.6)	-2.1 (-6.7, 2.6)	1.8 (-1.6, 5.3)	<b>2.6 (0.2, 5.1) *</b>	0.0 (-3.0, 2.9)
<i>DLOOP</i> Average <sup>1</sup>	<b>7.5 (4.8, 10.2) *</b>	0.7 (-5.4, 6.7)	0.6 (-2.6, 3.8)	-2.0 (-4.7, 0.8)	-2.6 (-7.2, 2.0)
<i>CYTB</i> CpG1 <sup>1</sup>	<b>7.9 (3.3, 12.5) *</b>	<b>3.4 (-0.2, 7.1) #</b>	1.1 (-1.0, 3.2)	<b>1.3 (-0.2, 2.7) #</b>	-0.8 (-2.2, 0.6)
<i>CYTB</i> CpG2 <sup>1</sup>	0.8 (-1.8, 3.4)	1.4 (-1.8, 4.5)	-0.2 (-1.9, 1.4)	0.6 (-0.7, 1.8)	<b>-0.8 (-1.0, -0.6) *</b>
<i>CYTB</i> CpG3 <sup>1</sup>	<b>3.9 (1.1, 6.6) *</b>	2.5 (-1.3, 6.2)	-0.1 (-2.3, 2.1)	0.6 (-1.2, 2.4)	-0.4 (-1.8, 0.9)
<i>CYTB</i> Average <sup>1</sup>	1.4 (-2.9, 5.6)	1.8 (-2.4, 6.0)	-0.2 (-2.5, 2.1)	0.8 (-1.1, 2.6)	-0.7 (-2.1, 0.6)
<i>MTTF</i> S1 CpG1 <sup>1</sup>	<b>5.9 (1.6, 10.3) *</b>	1.9 (-3.3, 7.0)	-1.1 (-4.2, 2.1)	-1.0 (-3.2, 1.2)	0.0 (-2.2, 2.1)
<i>MTTF</i> S2 CpG1 <sup>2</sup>	<b>-1.2 (-2.4, 0.0) *</b>	-0.3 (-2.3, 1.6)	-0.2 (-1.3, 0.8)	0.4 (-0.3, 1.1)	-0.7 (-1.6, 0.3)
<i>MTTF</i> S2 CpG2 <sup>2</sup>	<b>0.2 (0.0, 0.4) *</b>	-0.6 (-2.8, 1.5)	-0.6 (-1.6, 0.3)	-0.5 (-1.2, 0.3)	-0.1 (-2.4, 2.2)
<i>MTTF</i> S2 Average <sup>2</sup>	-1.5 (-3.4, 0.4)	-0.8 (-2.4, 0.8)	-0.4 (-1.5, 0.6)	0.0 (-0.9, 0.8)	-0.9 (-2.2, 0.4)

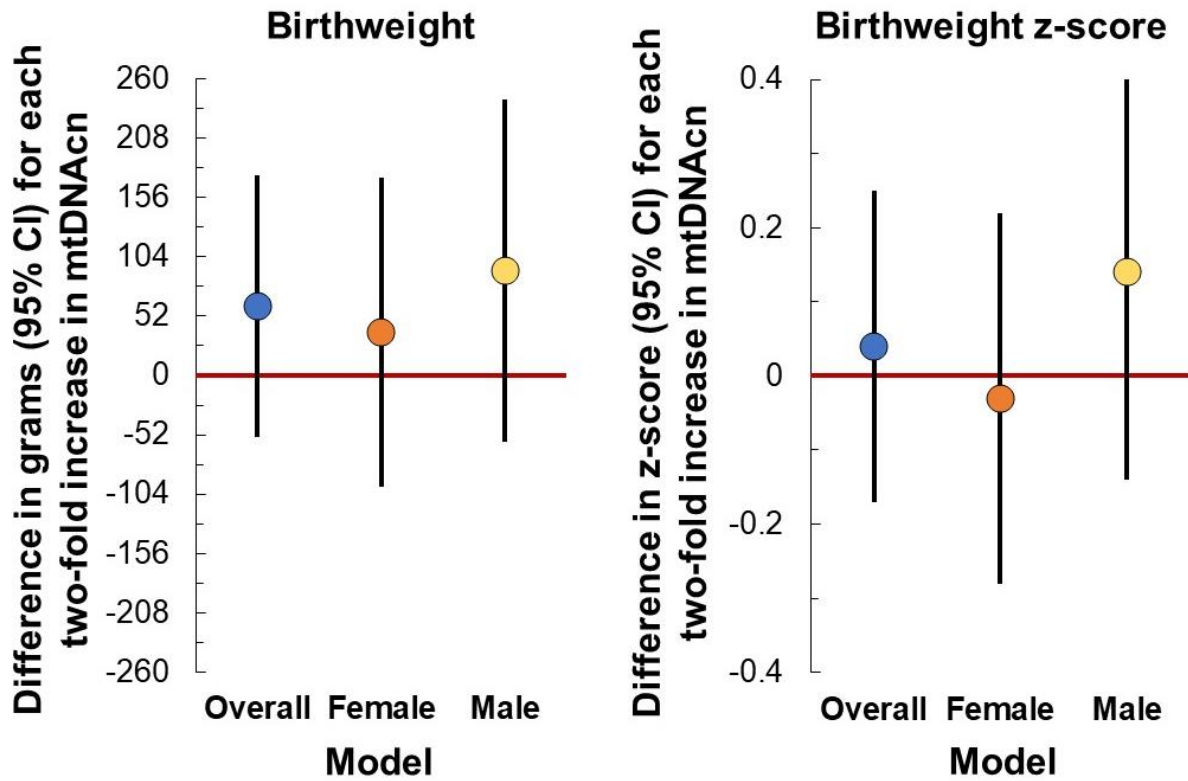
Covariate-adjusted quantile regression models accounted for plate number or hBG, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. **Bolded values are considered meaningful.** \* $p \leq 0.05$ ; # $p \leq 0.10$ .

<sup>1</sup>Gene sites were ln-transformed and  $\beta$  (95% CIs) were back-transformed using the following equation:  $\beta * \ln(2)$ . Results interpreted as the percent change in gestation length for each two-fold increase in mtDNA<sub>acn</sub> or mtDNA methylation.

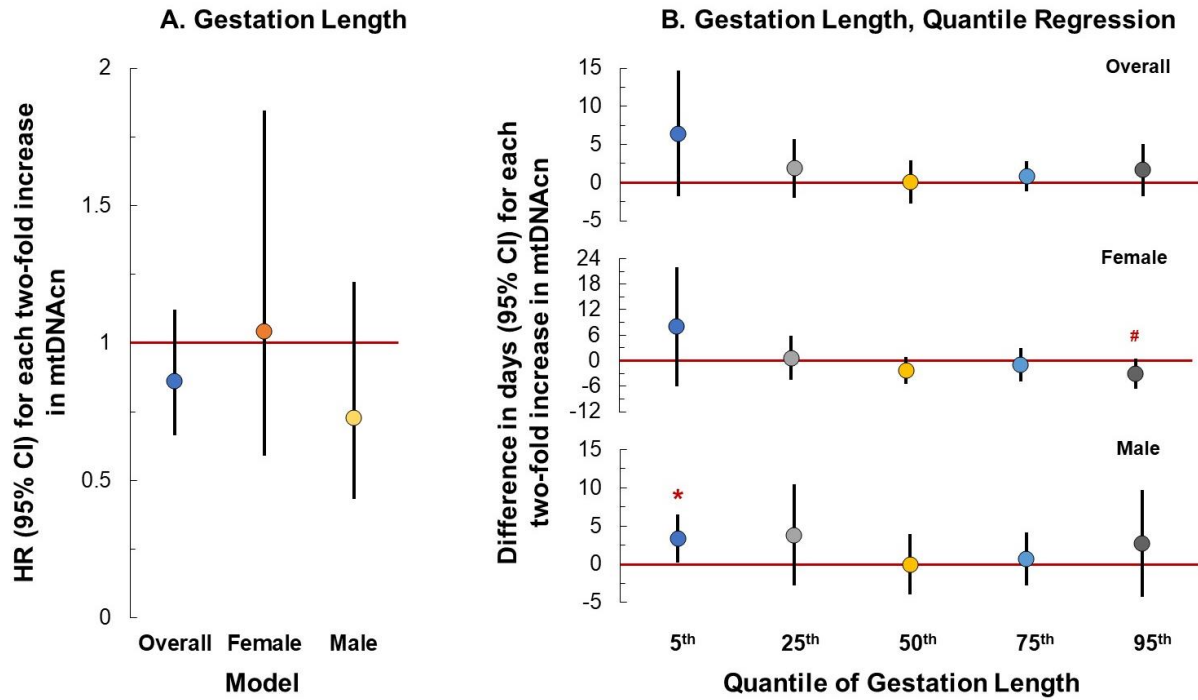
<sup>2</sup>Gene sites not transformed and  $\beta$  (95% CIs) were not adjusted. Results interpreted as the change in gestation length (in days) for each one-unit increase in mtDNA methylation.

BMI, body mass index; *CYTB*, cytochrome b; *DLOOP*, D-loop promoter region, heavy strand; *hBG*, human  $\beta$ -globin; *LDLR*, D-loop promoter region, light strand; *MTTF*, mitochondrially encoded tRNA phenylalanine; *NDI*, NADH-ubiquinone oxidoreductase chain 1; S1, location 1; S2, location 2.

## APPENDIX B: FIGURES



**Figure 1. Associations between mtDNAcn with birthweight and birthweight z-score using linear regression.** All models accounted for hBG, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. Results are back-transformed using the following equation:  $\beta \cdot \ln(2)$ . hBG, human  $\beta$ -globin; mtDNAcn, mitochondrial DNA copy number.



**Figure 2. Associations between mtDNAcn with gestational length evaluated using A) Cox PHMs and B) quantile regression.** All models accounted for hBG, race/ethnicity, income, age, tobacco use, pre-pregnancy BMI, week of bloodspot collection, parity, and fetal sex. Cox PHM results are back transformed using the following equation:  $e^{\log(\text{HR}) \cdot \log(2)}$ . Quantile regression results are back-transformed using the following equation:  $\beta \cdot \ln(2)$ . \* $p \leq 0.05$ ; # $p \leq 0.10$ . hBG, human  $\beta$ -globin; mtDNAcn, mitochondrial DNA copy number; PHM, proportional hazards model.