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Research Article

Maternal mitochondrial DNA copy number and methylation as possible predictors of pregnancy outcomes in a Michigan pregnancy cohort

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Abstract

Little is understood about the roles 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. 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* (mitochondrially encoded tRNA phenylalanine), *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. 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. 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.

Keywords: pregnancy; mitochondria; copy number; mtDNA methylation; gestational length; birthweight

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 USA, preterm birth is prevalent, being observed among 1 in every 10 infants [17]; furthermore, approximately 5%–15% of infants are born small for gestational age (SGA; birthweight < 10th percentile for gestational age) and 5%–20% of infants are born large for gestational age (LGA; birthweight > 90th 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.

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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 the site for conversion of 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 appropriate for gestational age, 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 subanalysis 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 nonpregnant populations, changes in mtDNA methylation have been associated with several diseases, including Alzheimer's disease, type 2 diabetes, and amyotrophic lateral sclerosis [35, 36]. Data on mtDNA methylation in pregnancy are limited, but it appears that environmental exposures may alter mtDNA methylation during this period. For example, in the USA, 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 $\leq 2.5\,\mu m$ ($PM_{2.5}$) 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 $PM_{2.5}$, reported that placental mtDNA methylation was inversely associated with birthweight, regardless of maternal smoking status and $PM_{2.5}$ 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.

Results

Characteristics of the analytic sample

Characteristics of the 396 eligible Archive for Research on Child Health (ARCH) participants are outlined in Table 1. The women in the analytic sample did not differ from the full sample (data not shown). The mothers in our study had a median age of 25 years (25th, 75th 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%).

Maternal mtDNA biomarker levels

All women had nonzero 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).

Distributions of birthweight, birthweight z-score, and gestational length

The distributions of birthweight, birthweight z-score, and gestational length are reported in Table 2. Briefly, the median (25th, 75th percentile) birthweight, birthweight z-score, and gestational length were 3345 grams (3005, 3643), –0.1 (–0.7, 0.6), and 276 days (269, 283), respectively. Approximately 8% of the infants in the sample were born preterm, and nearly one-third were born prior to 39 weeks (32%) (Table 2).

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 (Fig. 1, Supplementary Tables S1 and S2).

We observed no overall or fetal sex-specific associations of mtDNAcn with gestational length using Cox proportional hazards models (PHMs; Fig. 2a and Supplementary Table S3). Similarly, mtDNAcn was not associated with overall gestational length using quantile regression (Fig. 2b and Table 3); however, these associations differed by fetal sex. For instance, in females who were in the 95th percentile of gestational age, a two-fold increase in mtDNAcn was marginally associated with a 3.1-day [95% confidence interval (CI): –6.6, 0.5] shorter gestation (Fig. 2b and Table 3). Conversely, in males who were at the 5th percentile of gestational length, a two-fold increase in mtDNAcn was associated with a 3.3-day (95% CI: 0.2, 6.5) longer gestation (Fig. 2b and Table 3). In sensitivity analyses where we added a random day to obstetric-based estimates of gestation length (in weeks), quantile regression models

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

Characteristic	Analytic sample[a] ($n = 396$)
	Median (25th, 75th percentiles)
Age, years†	25.3 (21.8, 29.4)
Bloodspot collection, weeks†	11.0 (9.0, 14.0)
hBG†	0.6 (0.5, 0.7)
	n (%)
Race/ethnicity†	
White (ref.)	230 (58.1)
Other[b]	166 (41.9)
Annual household income†	
<$25 000	269 (67.9)
≥$25 000 (ref.)	127 (32.1)
Education	
Did not finish high school	63 (15.9)
High school or General Educational Development	124 (31.3)
Some college	128 (32.3)
College graduate or more	74 (18.7)
Marital status	
Married, living with baby's father	105 (26.5)
Married	17 (4.3)
Unmarried, living with baby's father	144 (36.4)
Unmarried	130 (32.8)
Tobacco use†	
No (ref.)	282 (71.2)
Yes	114 (28.8)
Pre-pregnancy BMI, categories†	
Do not have obesity (ref.)[c]	279 (70.5)
Have obesity[c]	117 (29.5)
Parity†	
0 children (ref.)	160 (40.4)
1+ children	236 (59.6)
Basis of gestational age estimate	
Last menstrual period	307 (77.5)
Obstetric	89 (22.5)
Fetal sex†	
Female (ref.)	202 (51.0)
Male	194 (49.0)
Size for gestational age	
Small	38 (9.6)
Appropriate	320 (80.8)
Large	38 (9.6)

An †indicates covariates included in the final model.
[a]Not all % equal 100. Missing from the analytic sample: education ($n = 7$).
[b]Other refers to Black or African American, American Indian, Alaska Native, Asian, Hawaiian/Pacific Islander or Hispanic.
[c]Do not have obesity: BMI < 30 kg/m^2; have obesity: BMI ≥ 30 kg/m^2.

remained consistent; however, associations at lower and upper quantiles became stronger, primarily driven by males and females, respectively (Supplementary Tables S4–S6).

Associations of mtDNA methylation with birthweight, birthweight z-score, and gestational length

In unadjusted linear regression models, maternal mtDNA methylation was not associated with birthweight or birthweight z-scores (Supplementary Tables S1 and S2). When adjusted for covariates, a two-fold increase in *CYTB* CpG3 percent methylation was associated with marginally higher birthweight (β: 66.5 grams, 95% CI: −9.7, 142.6) and birthweight z-score (β: 0.11, 95% CI: −0.03, 0.24). Additionally, a two-fold increase in *CYTB* CpG3 percent methylation was associated with marginally higher birthweight (β: 67.9 grams, 95% CI: −17.9, 153.6) (Table 4). A two-fold increase in *LDLR* CpG2 percent and *LDLR* CpG3 percent 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). When considering differences in associations by fetal sex, the association of *CYTB* CpG3 percent methylation with birth size was driven by males, such that a two-fold increase in percent 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). *LDLR* CpG2 methylation was associated with higher birthweight z-scores in females (β: 0.22, 95% CI: −0.02, 0.46) but not males, and the association of *LDLR* CpG3 percent methylation with birthweight z-score was driven by females (β: 0.19, 95% CI: 0.03, 0.42) (Table 4). Finally, a two-fold increase in *MTTF* S1 CpG1 percent 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 percent 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).

Overall, maternal mtDNA methylation was not associated with gestational length in crude or covariate-adjusted Cox PHMs (Table 4 and Supplementary Table S3). However, when we considered differences in associations by fetal sex, each two-fold increase in *DLOOP* CpG1% methylation was associated with a 30% lower hazard [hazard ratio (HR): 0.7, 95% CI: 0.4, 1.0] of birth before 39 weeks in females, and *MTTF S1* CpG1% methylation was associated with a 30% lower hazard (HR: 0.7, 95% CI: 0.5, 1.1; Table 4) in females. Using quantile regression, mtDNA methylation was associated with gestational length at its lower (5th) and higher (75th and 95th) percentiles (Table 3), which differed by fetal sex. Specifically, mtDNA methylation of several CpG sites was associated with longer gestation in females at the upper (50th, 75th, and 95th) percentiles (Table 3). Maternal mtDNA methylation of most CpG sites was generally associated with longer gestation in males in the lower (5th, 25th) percentiles, and shorter gestation at the highest (95th) percentile (Table 3). In sensitivity analyses where we added a random day to obstetric-based estimates of gestational length (in weeks), results from quantile regression models were generally consistent, but with more precision and generally stronger associations in the upper quantiles in males and females (Supplementary Tables S4–S6).

Discussion

Summary of major findings

In a sample of Midwestern US women with relatively lower socioeconomic status, several potential markers that could reflect 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, and measures of mitochondrial function therefore may have important clinical implications for pregnancy management. However, additional experimental studies are needed to establish the causal nature of our findings.

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, 40]. For instance, in pregnant Japanese

Table 2. Distribution of the mitochondrial DNA biomarkers and birth outcomes in the analytic sample ($n = 396$)

			Overall		Females		Males	
Gene and site	***n***	**% > 0**	**Median (25th, 75th) Percentiles**	**Mean (SD)**	**Median (25th, 75th) Percentiles**	**Mean (SD)**	**Median (25th, 75th) Percentiles**	**Mean (SD)**
ND1	396	100.0	0.6 (0.5, 0.7)	0.6 (0.0)	0.6 (0.5, 0.7)	0.6 (0.0)	0.6 (0.4, 0.7)	0.6 (0.0)
LDLR CpG1	351	86.5	2.8 (2.1, 3.6)	2.7 (0.1)	2.8 (1.9, 3.5)	2.6 (0.1)	2.8 (2.2, 3.7)	2.9 (0.1)
LDLR CpG2	316	18.5	0.0 (0.0, 0.0)	0.4 (0.1)	0.0 (0.0, 0.0)	0.3 (0.1)	0.0 (0.0, 0.0)	0.5 (0.1)
LDLR CpG3	196	47.4	0.0 (0.0, 2.2)	1.2 (0.1)	0.0 (0.0, 2.3)	1.2 (0.1)	1.0 (0.0, 2.2)	1.3 (0.2)
LDLR Average	351	86.8	1.4 (0.9, 2.2)	1.6 (0.1)	1.3 (0.8, 2.0)	1.4 (0.1)	1.5 (1.0, 2.3)	1.7 (0.1)
DLOOP CpG1	347	60.3	0.7 (0.0, 1.1)	0.8 (0.1)	0.7 (0.0, 1.1)	0.8 (0.1)	0.7 (0.0, 1.1)	0.9 (0.1)
DLOOP CpG2	304	40.3	0.0 (0.0, 0.9)	0.4 (0.0)	0.0 (0.0, 0.9)	0.4 (0.0)	0.0 (0.0, 0.8)	0.5 (0.1)
DLOOP CpG3	286	31.8	0.0 (0.0, 0.7)	0.4 (0.1)	0.0 (0.0, 0.7)	0.4 (0.1)	0.0 (0.0, 0.7)	0.4 (0.1)
DLOOP Average	347	61.5	0.3 (0.0, 0.9)	0.6 (0.0)	0.4 (0.0, 0.9)	0.5 (0.0)	0.4 (0.0, 0.9)	0.6 (0.1)
CYTB CpG1	306	32.6	0.0 (0.0, 1.8)	0.9 (0.1)	0.0 (0.0, 1.7)	0.9 (0.1)	0.0 (0.0, 1.8)	0.9 (0.1)
CYTB CpG2	295	36.5	0.0 (0.0, 2.6)	1.4 (0.1)	0.0 (0.0, 2.4)	1.1 (0.2)	0.0 (0.0, 2.7)	1.6 (0.2)
CYTB CpG3	291	18.7	0.0 (0.0, 0.0)	0.6 (0.1)	0.0 (0.0, 0.0)	0.6 (0.1)	0.0 (0.0, 0.0)	0.7 (0.1)
CYTB Average	306	49.0	0.0 (0.0, 1.2)	0.8 (0.1)	0.0 (0.0, 1.2)	0.8 (0.1)	0.4 (0.0, 1.2)	0.9 (0.1)
MTTF S1 CpG1	308	30.7	0.0 (0.0, 0.9)	0.5 (0.1)	0.0 (0.0, 1.0)	0.6 (0.1)	0.0 (0.0, 0.9)	0.5 (0.1)
MTTF S2 CpG1	298	89.2	4.2 (2.6, 5.1)	3.8 (0.1)	4.2 (2.7, 5.0)	3.8 (0.2)	4.2 (2.6, 5.2)	3.9 (0.2)
MTTF S2 CpG2	260	81.9	5.1 (3.6, 7.4)	5.3 (0.2)	5.5 (3.8, 7.5)	5.4 (0.2)	4.9 (3.5, 7.3)	5.2 (0.3)
MTTF S2 Average	298	93.2	4.7 (3.0, 5.7)	4.4 (0.1)	4.8 (2.9, 5.7)	4.4 (0.2)	4.6 (3.1, 5.6)	4.4 (0.2)
Outcome			Median (25th, 75th percentiles)					
Birthweight, grams			3345 (3005, 3643)		3343 (3005, 3629)		3345 (3005, 3646)	
Birthweight z-score[a]			−0.1 (−0.7, 0.6)		0.0 (−0.6, 0.7)		−0.2 (−0.8, 0.5)	
Gestational length, weeks[b]			39.4 (38.4, 40.4)		39.4 (38.7, 40.6)		39.3 (38.3, 40.1)	
Gestational length, days			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)	

[a]Birthweight for gestational age z-score was calculated using a US population-based reference [70].
[b]Gestation length was reported as the last menstrual period-based or obstetric-based estimate as determined using the algorithm by Basso and Wilcox [69].
S1, location 1; S2, location 2.

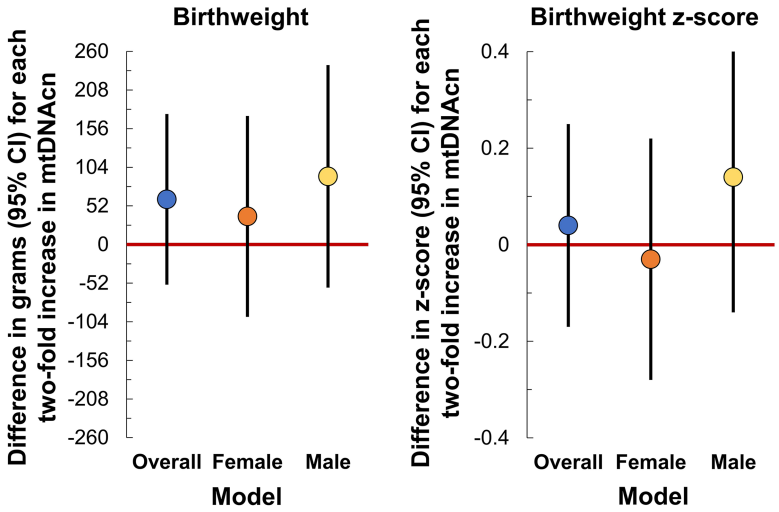

Figure 1. Associations between mtDNAcn and birthweight and birthweight z-score using linear regression; all models accounted for *hBG*, race/ethnicity, income, age, tobacco use, pre- pregnancy BMI, week of bloodspot collection, parity, and fetal sex; results are back-transformed using the following equation: β*ln(2).

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 (C_t value of *SLCO2B1* – C_t value of *ND1*) and (C_t value of *SERPINA1* – C_t value of *ND5*), was inversely associated with birthweight in males but not in females [40, 41]. 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 [42]. Beyond birthweight, several small case-control studies have reported associations of maternal peripheral mtDNA content [43] and placental mtDNA content [44, 45] 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 that 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) [46]. 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 week 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 [43, 47]. 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 directly 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 real-time quantitative polymerase chain reaction (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 ($n = 255$ nonsmokers, $n = 65$ former-smokers, and $n = 62$ smokers) enrolled in the ENVIRONAGE cohort, placental methylation of the nuclear DNA gene *CYP1A1* 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, 48]. Although the biological mechanisms driving the observed associations have not been elucidated, *CYTB* codes for the protein cytochrome b—a component of Complex III in the electron transport chain that

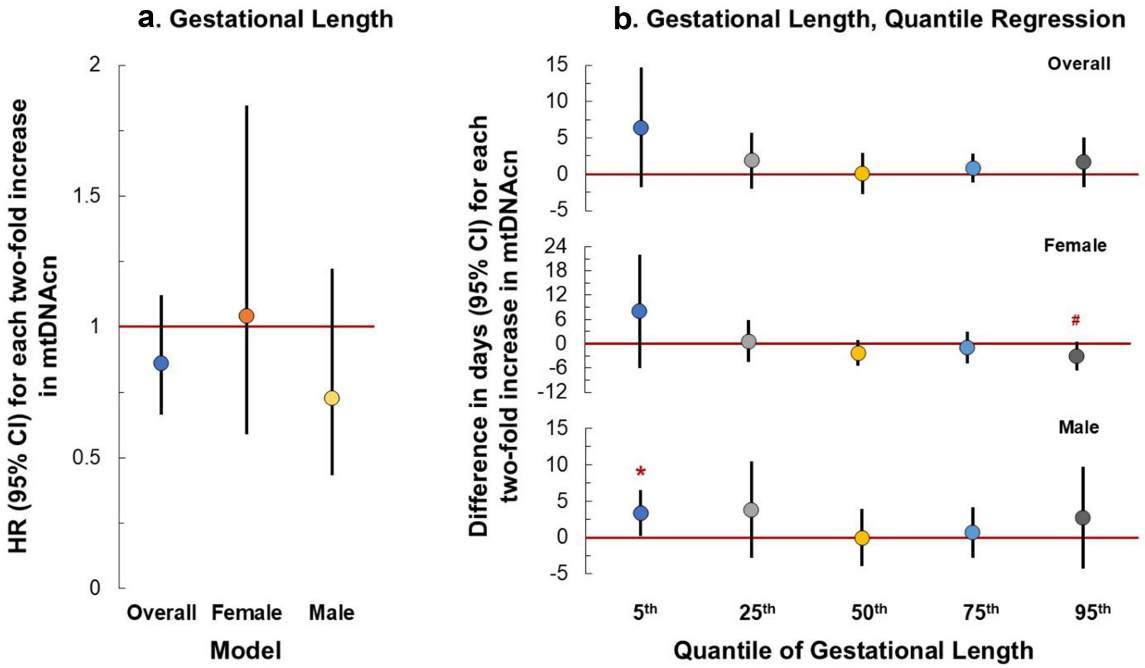

Figure 2. Associations between mtDNAcn and gestational length evaluated using (a) Cox proportional hazards model and (b) quantile regression; all models accounted for *hBG*, race/ethnicity, income, age, tobacco use, pre-pregnancy body mass index, week of bloodspot collection, parity, and fetal sex; Cox proportional hazards model results are back transformed using the following equation: $e^{\ln(HR)*\ln(2)}$ and quantile regression results are back-transformed using the following equation: $\beta*\ln(2)$; $^{*}P \leq .05$; $^{\#}P \leq .10$.

facilitates the transfer of electrons from ubiquinol to cytochrome c [37]—and the presence or absence of methylation may alter the function of *CYTB*, potentially disrupting the efficiency of the electron transport chain and adversely impacting fetal growth. Since gene expression was not measured, it is unclear if the magnitude of change observed here is enough to appreciably impact gene expression. Additionally, *MTTF* is a transfer RNA that is read during mitochondrial protein translation and altered methylation levels may impair the ability of the codon to be read during translation, thereby reducing mitochondrial efficiency and potentially impairing fetal growth [37, 49]. Alternatively, our observation that methylation of these genes was primarily associated with gestational length in newborns who were born either relatively early or later (in a sex-specific manner) may suggest methylation-mediated adaptations in metabolic processes that govern the length of pregnancy. Due to the plethora of functions performed by the mitochondria—including synthesis of sex-steroid hormones critical to pregnancy, further studies are needed to identify underlying mechanisms that are impacted by mtDNA methylation and further evaluate its capacity as a driver or biomarker of fetal growth.

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 [40]. 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 [50]. Overall, mitochondria-related gene expression was elevated in placentas of women who delivered preterm and post-term, though findings were inconsistent across cohorts [50]. 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. Although the role of mtDNA methylation remains unclear, mutations within and methylation of mtDNA may contribute to mitochondrial disease and mitophagy, thereby reducing mitochondrial function and inhibiting the body's ability to maintain homeostasis and respond to pregnancy [31, 36, 51]. As discussed previously, alterations to *CYTB* may reduce the efficiency of the electron transport chain, and these potential disruptions to maternal energy production may result in shortened gestation due to the inability to meet energy needs. Similarly, *DLOOP* and *LDLR* are responsible for mitochondrial DNA translation and regulation; atypical methylation may contribute to reduced mitochondrial efficiency and reduce gestational length by impeding the body's capacity to adapt to pregnancy [36, 37]. However, given our findings that association of mtDNA methylation with gestational

Table 3. Associations of maternal mtDNA and mtDNA methylation with gestational length by fetal sex, using quantile regression

Quantile		**5th percentile**	**25th percentile**	**50th percentile**	**75th percentile**	**95th percentile**
Gestational length, overall		**36.0 weeks**	**38.4 weeks**	**39.4 weeks**	**40.4 weeks**	**41.4 weeks**
Gestational length, female		**36.1 weeks**	**38.7 weeks**	**39.4 weeks**	**40.6 weeks**	**41.7 weeks**
Gestational length, male		**36.0 weeks**	**38.3 weeks**	**39.3 weeks**	**40.1 weeks**	**41.3 weeks**
Gene/site	**Model**	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
NDI[a]	Overall	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)
	Female	8.0 (−6.0, 22.1)	0.6 (−4.6, 5.8)	−2.3 (−5.4, 0.9)	−1.0 (−5.0, 3.0)	**−3.1 (−6.6, 0.4)****
	Male	**3.3 (0.2, 6.5)***	3.8 (−2.8, 10.4)	0.0 (−4.0, 4.0)	0.7 (−2.7, 4.1)	2.7 (−4.2, 9.7)
LDLR CpG1[a]	Overall	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)
	Female	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)
	Male	−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)
LDLR CpG2[a]	Overall	0.6 (−6.2, 7.3)	−1.2 (−4.8, 2.4)	−0.6 (−2.8, 1.6)	0.1 (−1.9, 2.0)	**1.9 (−0.1, 3.9)****
	Female	1.8 (−2.6, 6.3)	**−4.8 (−8.7, −1.0)***	−0.9 (−4.0, 2.2)	−1.6 (−4.9, 1.7)	**3.3 (1.4, 5.1)***
	Male	**−3.3 (−6.3, −0.3)***	0.5 (−5.0, 6.0)	−0.5 (−3.5, 2.5)	1.0 (−1.6, 3.7)	−2.7 (−8.6, 3.1)
LDLR CpG3[a]	Overall	−2.8 (−8.0, 2.4)	0.3 (−2.2, 2.8)	0.6 (−1.6, 2.7)	−0.4 (−1.8, 1.0)	**2.7 (0.9, 4.4)***
	Female	1.6 (−33.0, 36.2)	0.4 (−3.4, 4.2)	0.9 (−1.7, 3.6)	−0.8 (−3.2, 1.7)	**1.8 (0.1, 3.4)***
	Male	**2.6 (0.4, 4.8)***	0.2 (−4.4, 4.8)	−0.8 (−4.0, 2.4)	−0.9 (−3.5, 1.7)	0.3 (−5.1, 5.6)
LDLR Average[a]	Overall	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)
	Female	2.6 (−3.5, 8.8)	−2.0 (−6.3, 2.4)	−0.5 (−3.3, 2.3)	−1.7 (−4.6, 1.2)	**3.0 (1.0, 4.9)***
	Male	−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)
DLOOP CpG1[a]	Overall	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)
	Female	3.6 (−7.4, 14.6)	**3.9 (0.8, 6.9)***	**2.9 (0.2, 5.5)***	**3.4 (1.1, 5.6)***	1.9 (−1.0, 4.7)
	Male	**6.6 (3.3, 10.0)***	0.7 (−3.7, 5.0)	0.3 (−2.2, 2.7)	**−1.7 (−3.7, 0.2)****	**−2.8 (−5.8, 0.1)****
DLOOP CpG2[a]	Overall	5.1 (−2.9, 13.1)	1.2 (−2.1, 4.5)	0.9 (−1.5, 3.2)	1.1 (−0.8, 3.0)	**−2.7 (−5.6, 0.2)****
	Female	−5.8 (−30.4, 18.7)	0.4 (−4.3, 5.0)	1.6 (−1.5, 4.7)	0.8 (−2.9, 4.4)	**3.0 (−0.6, 6.6)****
	Male	−1.4 (−8.5, 5.7)	3.6 (−1.9, 9.1)	1.5 (−2.2, 5.2)	**2.8 (0.1, 5.6)***	−2.8 (−8.2, 2.7)
DLOOP CpG3[a]	Overall	−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)
	Female	−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)
	Male	−1.5 (−8.6, 5.6)	−2.1 (−6.7, 2.6)	1.8 (−1.6, 5.3)	**2.6 (0.2, 5.1)***	0.0 (−3.0, 2.9)
DLOOP Average[a]	Overall	4.8 (−1.1, 10.7)	1.2 (−2.0, 4.5)	1.1 (−1.3, 3.4)	0.8 (−1.2, 2.9)	**−1.9 (−3.8, 0.0)***
	Female	4.1 (−8.1, 16.4)	**4.1 (0.2, 8.1)***	**2.7 (−0.5, 5.8)****	**4.4 (0.7, 8.2)***	1.8 (−2.3, 5.9)
	Male	**7.5 (4.8, 10.2)***	0.7 (−5.4, 6.7)	0.6 (−2.6, 3.8)	−2.0 (−4.7, 0.8)	−2.6 (−7.2, 2.0)
CYTB CpG1[a]	Overall	**4.5 (1.7, 7.3)***	0.3 (−1.9, 2.5)	0.9 (−0.6, 2.4)	**0.9 (−0.1, 1.9)****	0.9 (−0.8, 2.6)
	Female	−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)
	Male	**7.9 (3.3, 12.5)***	**3.4 (−0.2, 7.1)****	1.1 (−1.0, 3.2)	**1.3 (−0.2, 2.7)****	−0.8 (−2.2, 0.6)
CYTB CpG2[a]	Overall	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)
	Female	3.0 (−7.2, 13.2)	−0.8 (−2.9, 1.2)	−0.8 (−2.5, 0.9)	0.4 (−1.4, 2.2)	**0.8 (0.1, 1.4)***
	Male	0.8 (−1.8, 3.4)	1.4 (−1.8, 4.5)	−0.2 (−1.9, 1.4)	0.6 (−0.7, 1.8)	**−0.8 (−1.0, −0.6)***
CYTB CpG3[a]	Overall	**2.3 (−0.2, 4.7)****	0.2 (−2.2, 2.6)	0.3 (−1.4, 2.0)	**1.0 (−0.2, 2.2)****	0.8 (−0.2, 1.7)
	Female	2.2 (−10.5, 14.9)	−0.8 (−3.4, 1.8)	**2.8 (0.3, 5.4)***	**2.3 (0.1, 4.5)***	1.6 (−0.5, 3.7)
	Male	**3.9 (1.1, 6.6)***	2.5 (−1.3, 6.2)	−0.1 (−2.3, 2.1)	0.6 (−1.2, 2.4)	−0.4 (−1.8, 0.9)
CYTB Average[a]	Overall	2.2 (−0.8, 5.3)	−0.3 (−2.8, 2.1)	0.4 (−1.4, 2.2)	**0.9 (−0.2, 2.0)****	1.2 (−0.5, 3.0)
	Female	3.0 (−16.1, 22.0)	−0.5 (−3.3, 2.2)	**2.0 (0.0, 4.0)***	1.2 (−0.9, 3.4)	**3.2 (0.5, 5.9)**
	Male	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)
MTTF S1 CpG1[a]	Overall	**8.2 (3.9, 12.5)***	1.9 (−0.7, 4.6)	1.0 (−1.0, 3.0)	−0.4 (−1.9, 1.2)	−0.8 (−2.6, 1.0)
	Female	**10.4 (4.8, 16.0)***	2.6 (−0.8, 6.0)	**3.1 (0.4, 5.8)***	1.1 (−1.1, 3.2)	0.0 (−3.4, 3.3)
	Male	**5.9 (1.6, 10.3)***	1.9 (−3.3, 7.0)	−1.1 (−4.2, 2.1)	−1.0 (−3.2, 1.2)	0.0 (−2.2, 2.1)
MTTF S2 CpG1[b]	Overall	−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)
	Female	−0.1 (−3.7, 3.6)	−0.4 (−2.0, 1.2)	−0.2 (−1.3, 0.9)	0.0 (−1.2, 1.1)	**1.1 (1.0, 1.3)***
	Male	**−1.2 (−2.4, 0.0)***	−0.3 (−2.3, 1.6)	−0.2 (−1.3, 0.8)	0.4 (−0.3, 1.1)	−0.7 (−1.6, 0.3)
MTTF S2 CpG2[b]	Overall	0.4 (−1.4, 2.1)	−0.6 (−1.8, 0.6)	−0.1 (−0.9, 0.7)	−0.3 (−0.9, 0.2)	**0.7 (−0.1, 1.5)****
	Female	0.4 (−11.7, 12.5)	−0.4 (−1.7, 0.9)	0.2 (−1.0, 1.4)	−0.3 (−1.2, 0.6)	**0.8 (0.5, 1.0)***
	Male	**0.2 (0.0, 0.4)***	−0.6 (−2.8, 1.5)	−0.6 (−1.6, 0.3)	−0.5 (−1.2, 0.3)	−0.1 (−2.4, 2.2)
MTTF S2 Average[b]	Overall	−1.1 (−2.9, 0.6)	−0.7 (−2.0, 0.5)	−0.4 (−1.1, 0.4)	0.1 (−0.5, 0.6)	**0.7 (0.0, 1.5)***
	Female	0.5 (−3.2, 4.1)	−0.7 (−2.2, 0.7)	−0.4 (−1.5, 0.7)	0.0 (−1.2, 1.1)	**0.9 (0.1, 1.7)***
	Male	−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.
*$P \leq .05$;
**$P \leq .10$.
[a]Gene sites were ln-transformed and β (95% CIs) were back-transformed using the following equation: β*ln(2). Results interpreted as the percent change in gestation length for each two-fold increase in mtDNAcn or mtDNA methylation.
[b]Gene sites not transformed and β (95% CIs) were not adjusted. Results interpreted as the change in gestation length (in days) for each one-unit increase in mtDNA methylation.
S1, location 1; S2, location 2.

Table 4. Associations of maternal mitochondrial DNA methylation with birthweight, birthweight z-score, and gestational length overall and by fetal sex, using linear regression and Cox PHMs

	Birthweight			Birthweight z-score			Gestational length		
	Overall, $n = 196–351$	Females, $n = 101–178$	Males, $n = 95–173$	Overall, $n = 196–351$	Females, $n = 101–178$	Males, $n = 95–173$	Overall, $n = 196–351$	Females, $n = 101–178$	Males, $n = 95–173$
Gene/site	**β (95% CI)**			**β (95% CI)**			**HR (95% CI)**		
LDLR CpG1[a]	26.2 (−54.0, 106.5)	45.7 (−71.0, 162.3)	10.7 (−94.4, 115.7)	0.07 (−0.08, 0.21)	0.10 (−0.11, 0.31)	0.04 (−0.15, 0.23)	1.1 (0.8, 1.4)	1.1 (0.8, 1.4)	1.1 (0.8, 1.5)
p-int		0.7			0.7				0.6
LDLR CpG2[a]	30.2 (−73.8, 134.1)	76.6 (−55.7, 208.9)	−34.6 (−189.0, 119.8)	**0.15 (−0.04, 0.33)**[**]	**0.22 (−0.02, 0.46)**[**]	0.05 (−0.23, 0.33)	1.1 (0.8, 1.5)	1.2 (0.8, 1.8)	1.1 (0.8, 1.6)
p-int		0.3			0.3				0.8
LDLR CpG3[a]	55.4 (−33.0, 143.8)	93.7 (−33.0, 220.5)	22.6 (−95.2, 140.3)	**0.14 (−0.01, 0.30)**[**]	**0.19 (−0.03, 0.42)**[**]	0.10 (−0.11, 0.31)	1.0 (0.8, 1.3)	1.0 (0.7, 1.4)	1.0 (0.7, 1.5)
p-int		0.4			0.5				0.9
LDLR Average[a]	43.7 (−55.5, 142.8)	76.0 (−58.4, 210.4)	11.6 (−122.2, 145.5)	0.11 (−0.07, 0.29)	0.16 (−0.08, 0.40)	0.05 (−0.19, 0.30)	1.1 (0.8, 1.5)	1.1 (0.7, 1.5)	1.1 (0.8, 1.6)
p-int		0.5			0.5				0.7
DLOOP CpG1[a]	23.0 (−58.6, 104.6)	34.4 (−73.9, 142.6)	8.2 (−114.8, 131.2)	−0.01 (−0.16, 0.14)	0.07 (−0.13, 0.27)	−0.13 (−0.35, 0.10)	0.8 (0.6, 1.1)	**0.7 (0.4, 1.0)**[*]	1.0 (0.7, 1.4)
p-int		0.6			0.2				**0.1**
DLOOP CpG2[a]	30.9 (−81.0, 142.7)	16.6 (−137.5, 170.6)	46.5 (−114.5, 207.5)	0.00 (−0.21, 0.21)	−0.04 (−0.33, 0.24)	0.05 (−0.26, 0.35)	1.0 (0.7, 1.4)	0.9 (0.5, 1.5)	1.1 (0.7, 1.7)
p-int		0.8			0.7				0.5
DLOOP CpG3[a]	11.4 (−99.6, 122.4)	−25.5 (−171.4, 120.4)	58.3 (−105.3, 221.9)	0.00 (−0.22, 0.22)	−0.08 (−0.37, 0.20)	0.11 (−0.21, 0.43)	1.0 (0.7, 1.5)	0.8 (0.4, 1.5)	1.1 (0.7, 1.8)
p-int		0.4			0.3				0.3
DLOOP Average[a]	42.4 (−62.6, 147.4)	29.6 (−112.6, 171.8)	57.2 (−95.0, 209.4)	0.01 (−0.18, 0.21)	0.04 (−0.22, 0.31)	−0.02 (−0.30, 0.26)	0.9 (0.6, 1.3)	0.7 (0.4, 1.1)	1.1 (0.7, 1.6)
p-int		0.8			0.7				**0.1**
CYTB CpG1[a]	14.8 (−53.8, 83.4)	47.4 (−49.3, 144.2)	−14.7 (−107.0, 77.6)	0.00 (−0.12, 0.12)	0.01 (−0.16, 0.18)	−0.01 (−0.17, 0.16)	1.0 (0.8, 1.3)	1.1 (0.8, 1.4)	0.9 (0.7, 1.3)
p-int		0.4			0.9				0.5
CYTB CpG2[a]	21.3 (−36.3, 78.8)	41.7 (−36.2, 119.6)	−3.1 (−88.2, 82.0)	0.02 (−0.08, 0.12)	0.08 (−0.06, 0.22)	−0.05 (−0.20, 0.10)	1.1 (0.9, 1.3)	1.1 (0.9, 1.4)	1.1 (0.9, 1.4)
p-int		0.4			**0.2**				0.8
CYTB CpG3[a]	**66.5 (−9.7, 142.6)**[**]	31.2 (−73.6, 136.0)	**105.3 (−4.6, 215.2)**[**]	**0.11 (−0.03, 0.24)**[**]	0.06 (−0.13, 0.25)	**0.16 (−0.03, 0.36)**[**]	1.0 (0.8, 1.3)	0.9 (0.7, 1.4)	1.0 (0.8, 1.4)
p-int		0.3			0.5				0.7
CYTB Average[a]	33.3 (−45.2, 111.9)	43.7 (−67.0, 154.4)	23.2 (−86.3, 132.7)	0.05 (−0.09, 0.19)	0.08 (−0.12, 0.28)	0.02 (−0.17, 0.22)	1.1 (0.8, 1.4)	1.1 (0.8, 1.5)	1.1 (0.8, 1.5)
p-int		0.8			0.7				1.0
MTTF S1 CpG1[a]	**67.9 (−17.9, 153.6)**[**]	−12.1 (−135.5, 111.3)	**143.2 (23.5, 262.9)**[*]	0.06 (−0.09, 0.22)	−0.05 (−0.27, 0.17)	**0.17 (−0.04, 0.38)**[**]	0.8 (0.6, 1.1)	**0.7 (0.5, 1.1)**[**]	0.9 (0.6, 1.4)
p-int		**0.1**			**0.2**				0.5
MTTF S2 CpG1[b]	16.6 (−18.5, 51.7)	16.8 (−29.7, 63.4)	16.3 (−32.5, 65.2)	0.04 (−0.02, 0.10)	0.03 (−0.05, 0.11)	0.06 (−0.03, 0.14)	1.0 (0.9, 1.1)	1.0 (0.9, 1.1)	1.0 (0.9, 1.1)
p-int		1.0			0.7				0.8
MTTF S2 CpG2[b]	10.9 (−24.4, 46.3)	9.8 (−31.1, 50.6)	12.5 (−32.6, 57.6)	0.04 (−0.03, 0.10)	0.03 (−0.05, 0.10)	0.05 (−0.03, 0.13)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)	1.0 (0.9, 1.2)
p-int		0.9			0.6				0.7
MTTF S2 Average[b]	10.4 (−24.6, 45.3)	6.2 (−38.4, 50.9)	15.0 (−31.6, 61.5)	**0.05 (−0.01, 0.11)**[**]	0.03 (−0.05, 0.11)	**0.07 (−0.01, 0.15)**[**]	1.1 (0.9, 1.2)	1.1 (0.9, 1.2)	1.1 (0.9, 1.2)
p-int		0.8			0.5				0.7

All linear regression models (birthweight and birthweight z-score) and Cox PHMs (gestational length) 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.

[*]$P \leq .05$;

[**]$P < .10$.

[a]Gene sites that are ln-transformed and β-estimates (95% CI) were back-transformed using the following equation: β*ln(2) for birthweight and birthweight z-score or $e^{\ln(HR)*\ln(2)}$ for gestational length. Results interpreted as the change in birthweight (g) or birthweight z-score for each two-fold increase in mtDNA methylation, or the percent change in the gestational length HR for each two-fold increase in mtDNA methylation.

[b]Gene sites not transformed; β-estimates (95% CI) and HRs (95% CIs) were not transformed. Results interpreted as the change in birthweight or birthweight z-score for each one-unit increase in mtDNA methylation or the HR for each one-unit increase in mtDNA methylation.

S1, location 1; S2, location 2.

age differs depending on the quantile of gestational age, additional experimental studies are needed to understand whether mtDNA methylation serves as an early clinical biomarker of pregnancy outcomes or as an underlying mechanism by which maternal metabolism adapts to support fetal development. 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. 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.

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 [52, 53]. Second, ARCH is composed of women from a Midwest US 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 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 directed acyclic graph (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, 54]. Despite this, 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 nonlinear associations were suspected. Seventh, the existence of mtDNA methylation has been largely debated in recent years due to the use of varied methodologies leading to inconsistent findings [31, 36, 55–57] and targeting CpG sites rather than non-CpG sites [56]. In our study, we quantified mtDNA methylation at CpG sites using bisulfite sequencing, which is considered the gold standard method due to its high sensitivity and reliability [30, 31]. We also linearized the mitochondrial DNA prior to analysis which has been shown to help reduce artifacts when performing bisulfite sequencing on this type of DNA. Importantly, other studies reported similar or higher levels of mtDNA methylation compared to those in our cohort. For example, the *ENVIRONAGE* cohort reported placental *DLOOP* methylation levels between 1% and 4% and levels of *LDLR* methylation between 3% and 10% in a sample of 60 placentas [31], and a study in Italian women reported *DLOOP* and *MTTF* methylation levels between 0.55% and 10.75% and 0%–14.75%, respectively [58]. Levels of *CYTB* methylation were not available in pregnant populations; however, the methylation level of *CYTB* from non-pregnant human blood samples was less than 3%, similar to our study [59]. Given other research, we anticipate that DNA methylation levels at non-CpG sites may be higher, but we were not able to profile these with the method we used. Finally, given 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 insight to the field by considering differences in fetal sex, a known key factor in fetal growth trajectory and overall gestational health.

Conclusions

In Midwestern US women with relatively low socioeconomic status, we observed that indirect 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 mtDNAcn 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.

Materials and methods

Recruitment and enrollment of participants into the ARCH pregnancy cohort and selection of the analytical sample

The current study used data from a subsample of pregnant women from 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 interviews, and clinical specimens (extra tubes of blood and urine collected beyond routine purposes) [60, 61]. The study has been described previously [61–63]. 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 [60]. 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) [61]. Eligible participants were ≥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 6 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 Supplementary Fig. S1. 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.

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 $25000", "$25000 to $49000", "$50000 to $74999," and "$75000 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 abstracted from birth certificates.

We calculated pre-pregnancy body mass index (BMI, in kg/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).

Maternal bloodspot collection and quantification of mtDNAcn

At the first prenatal appointment, we collected bloodspots from a maternal venous sample of whole blood. Specifically, up to five small (up to 0.5 cm^2 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 qRT-PCR [64, 65]. 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 [66]. To improve precision of our models, we also measured the nuclear gene human β-globin (*hBG*) using the following forward primer (hBG-F): 5′-TGCTGTCTCCATGTTTGATGTATCT-3′ and reverse primer (hBG-R): 3′-TCTCTGCTCCCCACCTCTAAGT-5′ [67]. The qPCR quantification of *ND1* and *hBG* 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. 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 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 (C_t) 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.

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. We duplicated one row of samples in every pyrosequencing batch, which reflects approximately 15% of the samples. Four mitochondria-specific genes relevant to pregnancy health and disease susceptibility were selected and their sequences amplified: *MTTF* (mitochondrially encoded tRNA phenylalanine) [58], *DLOOP* (D-loop promoter region, heavy strand) [31], *CYTB* (cytochrome b gene region) [68], and *LDLR* (D-loop promoter region, light strand) [31]. Both *DLOOP* and *LDLR* are located on the displacement loop, which does not overlap with nuclear DNA segments [31]. Primers were designed using the PyroMark Assay Design Software 2.0 (Qiagen) based on assays previously described by others [31, 58, 68] (Supplementary Table S7). mtDNA 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* (three 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.

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 [69, 70] 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 [69]. 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, the record would be discarded. If the LMP-based and obstetric-based estimates differed by more than 2 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 [70, 71]. 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 (Supplementary Fig. S2). 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 (≥39 weeks) gestation [72]. Newborn birthweights were abstracted from birth certificates, and we calculated sex-specific birthweight-for-gestational age z-scores according to published methods using a US population-based reference [70].

Statistical analysis

Selection of covariates

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 DAG (Supplementary Fig. S3) [27, 28, 73–76]. 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 *hBG* or methylation plate number, which were included as precision variables to account for potential differences in the laboratory environment and potential confounding due to batch effect [77]. 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 [78]: 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).

Descriptive statistics

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

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 *ND1* 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(mtDNA methylation + 1)] to avoid undefined estimates during transformation. Birthweight and birthweight z-score were not transformed. The resulting β-estimates and 95% CIs were back-transformed using the equation β*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 β-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 "Selection of covariates" section for the final covariate list) and checked regression diagnostics to ensure model assumptions were met.

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 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. This approach may also better model the true association because its improved statistical power (partly due to modeling gestational length as a continuous variable) increases its effectiveness, which is unlike logistic regression [79, 80]. 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 wk was defined as the event. Births ≥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 [81]. 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 [72, 82, 83]. 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 HRs and 95% CIs for these analyses were back-transformed using $e^{\ln(2)*\ln(HR)}$ to estimate the effect on the hazard ratio of a two-fold increase in mtDNAcn or mtDNA methylation. When each biomarker was not natural log-transformed, the estimate of its effect on the hazard ratio was for 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 nonlinear association of mtDNAcn or mtDNA methylation with gestational age at delivery. To understand if the association differed by fetal sex, we *a priori* stratified models by fetal sex. 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 outcome distributions are suspected to differ from association at the median [84–86]. We estimated confidence intervals using a rank-based approach [84–86]. Results are presented at the 5th, 25th, 50th, 75th, and 95th 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 nontransformed mtDNA methylation measures) at each quantile of gestational length. Quantile regression models accounted for covariates listed in "Selection of covariates" section.

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 [87, 88]. We did not adjust for multiple comparisons [89].

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 (Supplementary Table S3). 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 (Supplementary Tables S1–S3). 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 (Supplementary Tables S4–S6).

Supplementary data

Supplementary data is available at *EnvEpig* online.

Conflict of interest: None declared.

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