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Prenatal exposure to tobacco smoke leads to increased mitochondrial DNA content in umbilical cord serum associated to reduced gestational age

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Abstract

We investigated if prenatal exposures to tobacco smoke leads to changes in mitochondrial DNA content (mtDNA) in cord serum and adversely affect newborns health.

Umbilical cord serum cotinine levels were used to determine in-utero exposure to smoking. Cord serum mtDNA was measured by quantitative PCR analysis of the genes coding for cytochrome c oxidase1 (*MT-COI*) and cytochrome c oxidase2 (*MT-CO2*).

Log transformed levels of mtDNA coding for *MT-COI* and *MT-CO2* were significantly higher among infants of active smokers with higher serum level of cotinine ($p < 0.05$) and inversely associated with gestational age ($p = 0.08$; $p = 0.02$). Structural equation modeling results confirmed a positive association between cotinine and *MT-COI* and *MT-CO2* ($p < 0.01$) and inverse associations with gestational age ($p = 0.02$) and *IGF-1* ($p < 0.01$).

We identified a dose-dependent increase in the level of *MT-COI* and *MT-CO2* associated to increases cord serum cotinine and decreased gestational age.

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Keywords

Prenatal exposure; maternal smoking; mitochondrial DNA content; *MT-COI*; *MT-CO2*; molecular biomarkers in cord serum; gestational age

1. Introduction

Smoking exposure has been shown to be associated with variation in mitochondrial DNA (mtDNA) content and mitochondrial dysfunction in lung tissue, buccal cells and saliva of adult active smokers. Exposure to tobacco smoke is associated with an increase in buccal-cell and salivary mtDNA content in a dose-dependent fashion, for both smokers and former smokers, when compared with people who never smoked [1,2]. Masayesva and colleagues also showed a persistent high level of mtDNA content in saliva samples several decades after smoking cessation, which is consistent with a compensatory mechanism to the long term mtDNA damages caused by smoking [11]. Less is known about how maternal tobacco smoking may affect mtDNA in the fetus. Smoking during pregnancy is known to have serious consequences to the health of newborns, including loss of mitochondrial functionality [3,4]. Maternal smoking during pregnancy also is associated with placental mitochondrial dysfunction, which might contribute to restricted fetal growth by limiting energy availability in cells [5]. The primary aim of this study is to further understand the relationship between maternal smoking and mtDNA.

MtDNA is present at extremely high levels (10^3 to 10^4 copies per cell) in cells that are mostly homoplasmic (containing identical copies of MtDNA) at birth [6]. The human mitochondrial genome is small (16.5 kb) and encodes 13 genes of respiratory chain proteins, 22 genes for transfer RNA (tRNA), and two genes for ribosomal RNA (rRNA) (Supplementary Figure. S1). The proper functioning of mtDNA depends on its integrity but also on the number of copies, suggesting that even slight alterations in DNA sequences could have profound effects [6]. Moreover, altered mtDNA is associated with aging as well as smoking [7].

Due to the error-prone nature of mitochondrial replication and repair machinery, mtDNA is extremely vulnerable to damage; exposures to external mutagens or reactive oxygen species (ROS) cause a higher rate of mutations in mtDNA than in nuclear DNA [8]. Evidence suggests that mtDNA damage from oxidative stress accumulates over time [9], further reducing the integrity of mtDNA [10,11]. To compensate for the decline of respiratory function, the cell increases the mtDNA content [12]. Damage to mtDNA can cause injury to the respiratory chain, increasing production of ROS, thereby increasing mitochondrial mass and mtDNA content [13,14]. Smoking-related damage to respiratory chain function in lymphocytes is associated with measures of oxidative damage [15]. Smoking also inhibits mitochondrial enzyme activity in platelets and causes mitochondrial dysfunction in alveolar macrophages [16,17]

A secondary aim of this study is to further explore the relationship between maternal smoking, mtDNA and birth outcomes (growth and gestational age). Exposure to maternal smoking is known to be associated with a number of adverse birth outcomes, including

intrauterine growth restriction (IUGR) and preterm birth (PTB). PTB is defined as birth at less than 37 weeks gestation. Increasingly, studies of “late preterm” and “early term” pregnancies are identifying serious adverse health consequences along the continuum of gestational ages below 39 weeks of pregnancy [18–20]. IUGR occurs when fetal growth is small in proportion to gestational age. Maternal smoking, as measured by serum cotinine levels, has been associated with reduced weight, length and head circumference at birth [21]. Reduced size at birth is related to reduced gestational age, intrauterine growth restriction (IUGR), or both [22]. Preterm birth and reduced gestational age have been associated with maternal smoking [23]. IUGR has also long been associated with maternal smoking in pregnancy [24,25]. Increased mtDNA content has been found in placentas of newborns with an in-utero diagnosis of IUGR and in the blood of women carrying IUGR fetuses [26,27].

We hypothesized that *in-utero* exposure to maternal smoking, quantified in ng/ml of cotinine, would lead to increased mtDNA content in cord blood of newborns and be associated with impaired neonatal well-being, as measured by growth and gestational age. Cotinine, a metabolite of nicotine is used as an indicator of tobacco smoke exposure, including secondary smoke, because it is detectable for up to one week after the use of tobacco. The level of cotinine in the blood, saliva, and urine is proportionate to the amount of exposure to tobacco smoke. Specifically cotinine has been shown to be a sensitive and specific indicator for maternal exposure to active and second hand tobacco smoke. For these reasons cotinine is widely used as biomarker of smoke exposure [28] [29].

Exposure to maternal smoking is also associated with a decrease of Insulin-Like Growth factor 1 (*IGF-I*) in cord plasma and cord blood. *IGF-I* is known to play a fundamental role in fetal development, impacting proliferation, differentiation, and morphogenic pathways. *In utero* decreases in *IGF-I* have been linked to fetal growth impairment and reduced birth size [30,31]. Thus, we have sought to include umbilical serum *IGF-I* as a potential mediator for the relationship between maternal smoking, mtDNA and birth outcomes.

2. Materials and Methods

2.1 Study population

Subjects were selected from the Baltimore THREE (Tracking Health Related to Environmental Exposures) Study, a cross-sectional study of deliveries at the Johns Hopkins Hospital in Baltimore MD, designed to determine cord blood levels of a number of environmental chemicals, along with their relationship to thyroid hormone status and birth outcomes [32–35].

We collected umbilical cord blood from a sample of women presenting at the Johns Hopkins Hospital for delivery of singleton births from 26 November 2004 to 16 March 2005. Two study personnel abstracted demographic information and biological measures from the maternal medical records, and a 10% random sample was re-reviewed by study clinicians. Additional information was obtained from forms completed by the nursing staff at the time of delivery. Age, education, marital status, and parity were self-reported. Self-reported maternal race was also recorded because it appeared in the medical records as one of three categories: African American, Caucasian, and Asian. Smoking status was defined using the

maternal medical record. Gestational hypertension (GHTN) was categorized as present when the medical record included the diagnoses of pregnancy induced hypertension and/or preeclampsia.

There were 609 live births between November 2004 and March 2005. Of these, 597 were singleton births. We were able to collect cord blood for 341 of the singleton births, of which 41 were of insufficient quantity, leaving a total of 300 births eligible for the THREE study. Additionally, for this specific study, six births were excluded due to known major congenital anomalies, and one for an initial twin gestation, leaving a total of 293 study participants. Factors associated with insufficient blood volumes collected were preterm birth, low birth weight, being first born, and younger age of mother. Of these, 242 samples had sufficient serum volume for DNA isolation. However, high quality DNA was only available for confirmatory quantitative analyses for 142 samples, and these are the subject of this report.

Umbilical cord and umbilical cord blood samples were collected immediately after birth and stored at 4°C for less than 3 hours before further processing. Once collected, umbilical cord and cord blood samples were placed in the Labor and Delivery Suite refrigerators. Within a few hours of birth, samples were collected and transported by study personnel to a laboratory at the Johns Hopkins Bloomberg School of Public Health for processing. Umbilical cord samples were flash frozen. Blood specimens were centrifuged at 1000 g for 15 minutes, and serum was aliquoted into 2 ml polypropylene cryovials. Tissue and blood samples were then stored at -80 degrees Celsius.

Frozen samples were transferred on dry ice to the Tobacco Exposure Biomarkers laboratory at the Centers for Disease Control in Atlanta for cotinine analyses while other aliquots were archived. A total of 286 samples had sufficient volume for measurement of cotinine, which was conducted using positive-ion atmospheric pressure chemical ionization tandem mass spectrometry as previously described [36].

We analyzed serum *IGF-1* at the Bayview General Clinical Research Center using the Quantikine Human *IGF-1* Immunoassay, a solid-phase ELISA that contains E. coli-expressed recombinant human *IGF-1* (R&D Systems, Inc., Minneapolis, MN).

2.2 Human subjects

The study received approval from the Johns Hopkins Medicine Institutional Review Board and was determined to be HIPAA exempt. The IRB waived the requirement for informed consent because the collected biological samples would otherwise have been discarded and the sample collection constituted no more than minimal risk to the subjects. Strict procedures protected subject confidentiality and all data and specimens were anonymized. Members of a community advisory committee, who were selected for their specific knowledge, expertise and focus on important child health concerns in Maryland, had the opportunity to learn about and comment on this study before it was conducted.

2.3 DNA isolation from umbilical cord and umbilical cord blood serum

Twenty five milligrams of umbilical cord was cut up into small pieces and homogenized with a TissueRuptor (Qiagen) in the presence of 200 ul of lysis buffer for 3 minutes, as per

instructions in the DNeasy Blood & Tissue Kit (Qiagen). One ml of cord serum was obtained from each participant. Matched umbilical cord and umbilical cord serum samples were randomly sorted prior to DNA extraction, to reduce batch-processing bias, followed by digestion with 50 µg/ml proteinase K (Boehringer Mannheim, Germany) in the presence of 1% sodium dodecyl sulfate (SDS) at 48°C for 3 days, followed by phenol/chloroform extraction and ethanol precipitation and finally dissolved in 30 µl of LoTE (2.5 mmol/l EDTA and 10 mmol/l Tris-HCL) as previously described [37]. A total of 242 samples had sufficient serum volume for DNA isolation. However, high quality DNA from umbilical cord serum was only available for confirmatory quantitative analyses for 142 samples, and these are the subject of this report.

2.4 Quantitative polymerase chain reaction (PCR)

To measure the mtDNA content we performed real-time PCR of regions coding for mitochondrial genes, cytochrome *c* oxidase1 (*MT-CO1*) and cytochrome *c* oxidase2 (*MT-CO2*). The normalized dosage of these two mitochondrial subunit genes reflects the variation of mtDNA content [2,38]. A Perkin-Elmer/ABI 7900 thermocycler was used to perform real-time polymerase chain reaction (PCR) amplification for β -actin and mtDNA regions for *MT-CO1* and *MT-CO2* as previously described [39]. Primers were obtained from Invitrogen (Carlsbad, CA). The *MT-CO1* region was amplified using forward primer 5'-TTCGCCGACCGTTGACTATTCTCT-3' and reverse primer 5'-AAGATTATTACAAATGCATGGGC. *MT-CO2* region amplification was done using forward primer 5'-CCCCACATTAGGCTTAAAAACAGAT-3V and reverse primer 5VTATACCCCCGGTCGTGTAGC-3V. β -Actin amplification was achieved using forward primer 5'-ACCCACACTGTGCCCATCTAC-3' and reverse primer 5'-TCGGTGAGGATCTTCATGAGGTA-3'. All TaqMan probes (Applied Biosystems, Foster City, CA) were 5'-FAM and 3'-TAMRA labeled. *MT-CO1* probe 6-FAM-AACGACCACATCTACAACGTTATCGTCAC-TAMRA and β -actin probe 6-FAM-ATGCCCTCCCCCATGCCATCC-TAMRA were used. Probe and primer combinations were designed to avoid amplification of pseudo genes, by performing BLAST analysis of primers and probes. PCR amplifications were carried out in buffer containing 16.6 mmol/l ammonium sulfate, 67 mmol/l Trizma, 2.5 mmol/l MgCl₂, 10 mmol/l β -mercaptoethanol, 0.1% dimethyl sulfoxide, 600 nmol/l each of forward and reverse primers, 200 nmol/l TaqMan probe, 0.6 unit of platinum Taq polymerase, and 2% Rox reference dye. Five hundred picograms of DNA were used to amplify mitochondrial regions, whereas 10 ng were used to amplify β -actin, a single copy gene. The real-time PCR reactions were performed in triplicate for each gene. Standard amplification curves for *MT-CO1*, *MT-CO2* and β -actin genes were obtained using JHU-O11 cell line DNA. Mitochondrial to nuclear DNA ratios were calculated by dividing the mtDNA signal for each gene by the corresponding β -actin signal.

2.5 Statistical analysis

For the umbilical cord serum analyses, we compared mtDNA coding for *MT-CO1* and *MT-CO2* levels between newborns born to maternal smokers versus maternal non-smokers (by self-report) and, classified, according to the levels of cord blood serum cotinine levels. Levels >10 ng/ml considered "high" or consistent with active smoking and levels <1 ng/ml

considered “low” or consistent with non-smoking. There was some difference in the classification of smoking by cotinine levels versus the medical record; two mothers reporting smoking during pregnancy had infants whose cord serum cotinine levels were <1 ng/ml (0.871 and 0.115 ng/ml). Five mothers not reporting smoking had infants with cord serum cotinine levels within the active smoking range (>10 ng/ml). We evaluated mtDNA content in paired umbilical cord and cord serum samples from newborns exposed to very low levels of cotinine in utero (Non-Smokers n=106; mean = 0.05 ng/ml) and newborns exposed to high levels of cord serum cotinine. (Active and Passive Smokers n=36; mean = 12.1 ng/ml).

Because mtDNA coding for *MT-COI* and *MT-CO2*, as well as cotinine, were log-normally distributed, we log transformed them for statistical analyses and computed medians and geometric means rather than arithmetic means. Linear regression analyses were used to assess relationships between the natural log of mtDNA coding for *MT-COI* and *MT-CO2* versus both measures of smoking (the natural log of cotinine and self-reported smoking), as well as measures of maternal and neonatal health, namely, GHTN, pregnancy-related diabetes, gestational age, birthweight, length, and head circumference for gestational age. (Analyses were repeated without log transforming the levels of mtDNA coding for *MT-COI* and *MT-CO2* with essentially the same results, not shown in this paper)

Bivariate data analyses were conducted to identify potential confounders for relationships among exposure: cotinine; intermediate outcomes: mtDNA coding for *MT-COI* and *MT-CO2* and *IGF-I*; birth outcomes (gestational age, length, head circumference and birthweight); and demographic variables.

First-generation multivariate regression modeling is an excellent tool to predict variance in a dependent variable, based on linear combinations of independent variables. However, when evaluating more complex relationships among variables in which variables other than the independent variable are, themselves, interrelated, first-generation models are not appropriate. As described in our results, this is the case for several important relationships that we studied which found both direct and indirect pathways that explain the relationships between smoking and birth outcomes. First generation models fail to capture such indirect as well as direct pathways. Therefore, structural equation modeling (SEM) was used to assess complex interrelationships among exposures, outcomes and potential confounders/modifying factors. SEM, more commonly used in the social sciences, is a statistical tool that enables the construction of complex directed acyclic graphs (DAGs) for conceptualizing complex models and the use of regression analysis to test such models [40]. SEMs extend standard multiple regression techniques through a system of linear equations that define the statistical relationships among measured variables to allow for the evaluation of more complex relationships than more commonly employed multiple regression techniques. Covariates—like sex or race—can be incorporated into SEM analysis. SEM can be used to model not only direct effects, but also mediating effects, such as effects that occur when one variable affects another variable, which in turn affects a third. As described by Beran, et al, SEM modeling is straightforward but this methodology has some of the same limitations as the underlying multiple regression techniques that they incorporate [41]. Structural equation modeling provides a framework for statistical analysis that includes multivariate procedures, and has been used in other research to assess complex genetic and environmental

relationships [42] complex physiological interactions [43] and even complex public opinion data [44].

Stata version 13 was used to construct a hypothesized version of the graphical SEM and to perform statistical tests for direct and indirect effects, estimated parameters and combinations of estimated parameters [45]. The SEM estimated the effect of exposure to maternal smoking (determined by umbilical cord serum cotinine levels as a continuous independent variable) on birth outcomes (gestational age and head circumference as continuous dependent variables). Levels of mtDNA coding for *MT-CO1* and *MT-CO2*, separately, as well as umbilical cord serum *IGF-1*, were modeled as continuous intervening variables. Several possible confounding variables for these relationships were controlled for in the model as categorical variables: sex, maternal race (African American), maternal age (<18), prepregnancy BMI (underweight), prepregnancy BMI (obese), GHTN, parity (one or more prior pregnancy), and medical coverage (private insurance). Final models did not include maternal age, BMI (underweight), parity or medical coverage. Parity had a slightly poorer fit than race (African American) and its inclusion (in the place of race) would have led to similar conclusions. For all variables in the final model, we calculated both direct effects and total effects on birth outcomes. Direct effects were the coefficients of exogenous variables on dependent variables, conditional on all other variables in the equation. Total effects computed the coefficient of exogenous variables on dependent variables after accounting for all the simultaneity in the system. Total effects include not only direct but also indirect (mediating) effects.

3. Results

Among the 142 newborns included in this study, 78 were male and 64 were female. The 142 newborns included in this study were similar to all 300 babies in the THREE study cohort (S1 Table) with respect to demographics, smoking status and cotinine levels. Maternal age ranged from 14 to 40 years and paternal age from 14 to 43 years. Twelve mothers were <18 years of age and 11 were >35. The majority of mothers, 103 (72%) were African American, 30 (21%) Caucasian and 9 (6%) Asian. This ratio was similar to the ratio of African American babies in the entire 300-person study (69%) and the hospital as a whole for that year (70%). The average birth weight was 3193g (SD 578g), somewhat higher for African Americans (3236 ± SD 548g) than Caucasians (3101 ± SD 671g) and Asians (3004 ± SD 581g). These data reflect the unique demographics of the population delivering infants at this hospital during this time period and are not generalizable.

We found higher levels of mtDNA coding for *MT-CO1* and *MT-CO2* in cord blood serum DNA of newborns exposed to high cotinine levels when compared to newborns exposed of very low cotinine levels in cord serum (*MT-CO1* p=0.02, *MT-CO2* p=0.02), as can be seen in Figure 1. The cord serum levels of *MT-CO1* and *MT-CO2* mtDNA were highly correlated (0.95), as can be seen in the scatter plots in Supplementary Figure. S2 a and b, respectively.

To evaluate the relationship between *MT-CO1* and *MT-CO2* and smoking we assessed relationships with possible confounding variables (Tables 1a and 1b). Log transformed levels of mtDNA coding for *MT-CO1* and *MT-CO2* were higher for male infants (*MT-CO1*

$p=0.006$; $MT-CO2$ $p=0.008$), for infants of mothers with prepregnancy BMI classified as obese ($MT-CO1$ $p=0.06$ and $MT-CO2$ $p=0.07$); for Infants from pregnancies complicated by GHTN ($MT-CO1$ $p=0.05$ and $MT-CO2$ $p=0.18$); and for infants of parous mothers (women with at least one prior pregnancy) ($MT-CO1$ $p=0.07$ and $MT-CO2$ $p=0.02$). We did not observe associations between the values of mtDNA coding for $MT-CO1$ or $MT-CO2$ with maternal age, maternal diabetes, medical insurance status (a measure of socioeconomic status), BMI as a continuous variable, or infant birthweight, head circumference and length. Cotinine (log transformed) values (Tables 1a and 1b) were, on the other hand, significantly higher in African American mothers ($p=0.0002$); women with public insurance ($p<0.0001$); youngest mothers (<18 years, $p=0.10$); infants of obese mothers; and mothers with prior pregnancies ($p=0.08$). Cotinine levels were lower among mothers >35 years of age ($p=0.12$). Maternal diabetes was not associated with cotinine (Table 1a). There was little evidence for confounding given that no variables were associated with both $MT-CO1$ and $MT-CO2$ and cotinine. Multivariate models separately regressing cotinine on $MT-CO1$ and $MT-CO2$, controlling for infant sex, maternal parity, and race showed strong positive linear relationships between the natural logs of $MT-CO1$ and $MT-CO2$ and cotinine ($p=0.014$ and $p=0.007$ respectively).

To evaluate possible relationships between maternal smoking, mtDNA and birth outcomes, we evaluated relationships between birth outcomes and other variables (Tables 1b and 1c). As shown in Table 1b, gestational age is strongly associated with birthweight, length and head circumference and each of these three growth parameters is strongly correlated with all of the others. Cotinine is negatively associated with gestational age and all three growth-measures, as well as $IGF-1$, and positively associated with $MT-CO1$ and $MT-CO2$. $IGF-1$ is positively correlated with all birth outcomes and negatively with cotinine. $MT-CO1$ and $MT-CO2$ are strongly correlated. Both are negatively correlated with gestational age.

Because gestational age is not only a birth outcome, but also a strong predictor of growth measures, we evaluated bivariate associations between exposures and potential modifying and confounding factors on growth measures, while controlling for gestational age (Table 1c).

Reduced gestational age was most strongly associated with GHTN, serum cotinine levels and $MT-CO1$ and $MT-CO2$ levels. Male babies had lower birthweight and length for gestational age. Cotinine levels were associated with reduced gestational age and smaller head circumference for gestational age. $IGF-1$ was associated with increased gestational age. The levels mtDNA coding for $MT-CO1$ and $MT-CO2$ were negatively associated with gestational age and positively associated with head circumference (more strongly for $MT-CO2$). These associations with head circumference only appear after controlling for gestational age (Table 1b) suggesting that the head circumference is larger for gestational age with increasing $MT-CO1$ and $MT-CO2$ levels.

In an earlier publication [32], we found that delivery mode by C-section and maternal height were associated with head circumference; however, these variables were not associated with levels of mtDNA coding for $MT-CO1$, $MT-CO2$ nor with cotinine, and these data are not included nor were these variables included in models.

The final SEM models of mtDNA levels coding for *MT-CO1* and *MT-CO2* are shown in Figure 2. The strength of statistical associations is shown giving the standardized adjusted regression coefficients for each path in models. Likelihood ratios and probabilities greater than chi-square were for each model respectively, 19.96 and 0.28 and 18.43 and 0.43. Figure 2 demonstrates individual relationships among the variables included in SEM models; total relationships are summarized in Table 2. As shown in Figure 2, cotinine had direct positive associations with mtDNA coding for both *MT-CO1* and *MT-CO2*. On the other hand, cotinine had negative associations with both gestational age and head circumference. Cotinine also had a negative association with *IGF-1*, which, in turn, had a direct positive association with head circumference. Figure 2 also shows how the SEM controls for potential confounding by maternal race, sex and other variables. As would be expected, longer gestational age was associated with having a larger head circumference.

The final results of SEM modeling are summarized in Table 2, which displays the total (direct + indirect) non-standardized associations for key variables in the pathway from cotinine to gestational age and birth weight, length and head circumference. Summarizing direct and indirect effects together, cotinine had strong negative associations with *IGF-1* ($p < 0.01$); strong positive associations with levels of mtDNA coding for both *MT-CO1* and *MT-CO2* ($p < 0.01$ and $p < 0.001$, respectively); and strong negative associations with both gestational age ($p < 0.05$) and head circumference ($p < 0.05$). *IGF-1* had a strong positive total (direct + indirect) association with head circumference ($p = 0.01$). Levels of mtDNA coding for both *MT-CO1* and *MT-CO2* had a strong total direct + indirect associations with gestational age (-3 days with each log increase in *MT-CO1* and -3.7 days with each log increase in *MT-CO2*, $p < 0.05$). For *MT-CO1* and *MT-CO2* there was evidence for an increase in head circumference (0.2 cm with each log increase in *MT-CO1* and 0.3 cm with each log increase in *MT-CO2*, $p = 0.08$ and 0.09 respectively). More detailed SEM results can be seen in S2 Table.

4. Discussion

Our results show an increase of mtDNA coding for *MT-CO1* and *MT-CO2* in newborns whose mothers were smokers or exposed to second-hand smoke during pregnancy compared to newborns with non-smoking mothers.

Higher levels of mtDNA coding for *MT-CO1* and *MT-CO2* were associated with reduced gestational age. Because smoking is a known risk factor for reduced gestational age and intrauterine growth, we used structural equation modeling to dissect the interrelationships between smoking (as assessed by cotinine levels), abundance of mtDNA coding for *MT-CO1* and *MT-CO2*, *IGF-1*, and birth outcomes. SEM confirmed that there are strong independent associations between cotinine exposure levels and higher levels of mtDNA coding for *MT-CO1* and *MT-CO2*, which are in turn associated with reductions in gestational age. Therefore, it seems possible from these results that mtDNA content not only may be part of the pathway for smoking's negative effect on gestational age but may also, in itself, be involved with reduced gestational age. On the other hand, SEM did not find an independent effect of *MT-CO1* and *MT-CO2* copy number on intrauterine growth restriction, i.e., reduced birthweight, length and head circumference for gestational age, but in our final

SEM there was some evidence for an association between *MT-COI* and *MT-CO2* copy number and increased head circumference for gestational age. The final SEM shows also an association between cotinine and reduced levels of *IGF-1*, which is independently associated with head circumference but not gestational age.

We have identified a potential pathway for cigarette smoke constituents' adverse effect on newborns involving a dose-dependent increase in mtDNA coding for *MT-COI* and *MT-CO2* levels in cord blood serum linked to decreased gestational age. This result is consistent with Masayesva et al. [2] who reported a dose dependent association of smoking with an increase in mtDNA coding for *MT-COI* and *MT-CO2* in saliva. Other studies show that smoking during pregnancy may lead to variations in the number of mtDNA copies and dysfunction of mitochondria on other tissues such as the placenta, and that there is an association with reduced gestational age. Some of these studies reported a negative correlation with the number of cigarettes per day and the mtDNA content in the placenta, hypothetically due to damages of the mitochondrial DNA replication and regulatory mechanisms by tobacco toxic chemicals[5]. On the contrary, other studies report an increase of mtDNA content in IUGR placenta possibly as a compensatory mechanism for the decline of respiratory function due to smoke's damages [26, 12]. Unfortunately, these studies have a limited number of participants and their results besides being contradictory, are inconclusive.

SEM results also suggest the implication of a second pathway involving *IGF-1* and resulting in decreased growth (head circumference) for gestational age. Earlier studies have associated smoking with reduced levels of *IGF-1* and reduced birth size [30,31]. Ours is the first to evaluate these relationships with a structural equation model that assessed possible exposure and intermediate pathway interactions that may explain some of the deleterious effects caused by prenatal exposure to tobacco smoking. Additional studies will be needed to confirm these findings.

Prenatal exposure to tobacco smoke causes mutagenic and oxidative damage that leads to respiratory infections, asthma, allergies, and neurobehavioral complications. [46–53]. Epidemiological and molecular studies reported also a possible increase in risk of childhood cancer (brain tumors and acute lymphoblastic leukemia in particular) in association with prenatal exposure to tobacco smoke, although this association is still controversial [54–60]. Since the oxidative phosphorylation mechanism is fundamental in aerobic metabolism, it is not surprising that exposure to oxidative or mutagenic agents may damage the respiratory chain and disrupt oxidative phosphorylation.

Studies report that mtDNA is more susceptible to oxidative and mutagenic damage compared to the nuclear genome [51,61]. This high susceptibility to damage may be linked to the absence of histones, to the error-prone nature of the mitochondrial replication and repair machinery, as well as to the production of a large amount of reactive oxygen species and the proximity of accumulated toxic xenobiotics to mtDNA [8,62]. MtDNA damage reduces the functionality of the respiratory chain and increases the production of ROS, giving rise to a vicious circle. Thus, cells increase mitochondrial mass and mtDNA content in order to compensate for the decreased production of energy [2,16,17,63,64]. This mechanism of response to the action of genotoxic agents or oxidants, such as smoking,

could be an indicator of altered mitochondrial function, and explain the increase in mtDNA content in cord serum.

We found levels of mtDNA coding for *MT-COI* and *MT-CO2* were associated with cotinine and lower gestational age. There are several ways to explain this association. Maternal smoking may be affecting the functionality of the respiratory chain in utero, whether via mutational or oxidative damage directly to fetal mtDNA and/or via alternative effects on oxygen delivery. Tobacco smoke contains several oxidative and mutagenic substances that affect the functionality of the respiratory chain or lead to mutations in mtDNA [65]. This reduction in gestational age, while small, is a significant effect on the fetus across the population. Pregnant smokers have elevated oxygen radical injury that can be measured by breath ethane, produced by peroxidation of omega-3-fatty acids. Schwartz and colleagues showed that ethane was elevated in pregnant smokers when compared to pregnant non-smokers, suggesting that cigarette smoking is therefore pro-oxidant [66]. Longo published one of several studies reporting associations between smoking during pregnancy and decreased oxygen delivery to the fetus [67]. Of the numerous compounds in cigarette smoke, there are three prime candidates for producing this effect: nicotine, of which cotinine is a stable metabolite, carbon monoxide and cyanide. Nicotine and carbon monoxide are found in higher concentrations in cord blood than in maternal blood, as is the metabolite of cyanide, thiocyanate. Nicotine is believed to act by producing vasospasm resulting in poorer placental perfusion [68,69] while carbon monoxide and cyanide are both direct respiratory poisons [67,70]. In our study, cotinine is a direct measure of nicotine but also is a marker for other harmful constituents of tobacco smoke. Additional studies will be required to elucidate how maternal smoking may be affecting the functionality of the respiratory chain in utero, whether via mutational or oxidative damage directly to fetal mtDNA and/or via alternative effects on oxygen delivery and energy metabolism.

The role of mitochondria in energy production is critically important for placental function during embryonic development. Impaired mitochondrial function has been shown to lead to low birthweight in mice [71]. Other studies have described the association between mtDNA content and mitochondrial protein mutations with IUGR [26,72]. Our SEM results, showing *IGF-1* had a direct positive association with levels of mtDNA coding for *MT-COI* and *MT-CO2*, contributes to accumulating evidence that mitochondria play a central role in fetal metabolic programming [73]. While this paper was under revision, two manuscripts were published providing more evidence of the dynamic relationship between prenatal exposure, mtDNA content, IUGR and metabolic programming in utero [74]. Effective fetal growth is dependent on the transport of nutrients from the placenta to the fetus, which requires ATP, insulin, and several growth factors, including *IGF-1*. Changes in mitochondrial morphology detected with high throughput imaging suggest that placental mitochondrial function is a regulator of fetal and placental growth [75]. However, our results suggest that mitochondrial function may be linked to adverse birth outcomes via earlier gestational age rather than via intrauterine growth restriction. Nonetheless, the accumulating evidence suggests that metabolic programming by prenatal exposures is mediated by alterations in mitochondrial structural alterations, which in turn leads to changes in mitochondrial function and content, to maintain homeostasis in energy production. Together this evidence indicates that mitochondria play an important role in the fetal metabolic response to adverse prenatal

exposures and may be at the mechanistic center of pathways linking these exposures to adverse health outcomes throughout the life course [76].

This study was limited by the availability of umbilical cord blood serum of enough quality and quantity to extract circulating fetal DNA that could be used for downstream analyses. For the same reason, it was not feasible to compare the quantity of mtDNA with mitochondrial mass. Another limitation of the study was that maternal blood was not collected during the study, negating the possibility of establishing a link between maternal and fetal serum cotinine levels. Moreover, other environmental and hereditary factors may influence the exposure to damaging agents and alter the mitochondrial response to tobacco. Notwithstanding these limitations, the observed effects are consistent with prior observations made by several investigators, providing biological plausibility for the results we are reporting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Tan D, Goerlitz DS, Dumitrescu RG, Han D, Seillier-Moiseiwitsch F, et al. Associations between cigarette smoking and mitochondrial DNA abnormalities in buccal cells. *Carcinogenesis*. 2008; 29:1170–1177. [PubMed: 18281252]
2. Masayeva BG, Mambo E, Taylor RJ, Goloubeva OG, Zhou S, et al. Mitochondrial DNA content increase in response to cigarette smoking. *Cancer Epidemiol Biomarkers Prev*. 2006; 15:19–24. [PubMed: 16434581]
3. Hackshaw A, Rodeck C, Boniface S. Maternal smoking in pregnancy and birth defects: a systematic review based on 173 687 malformed cases and 11.7 million controls. *Hum Reprod Update*. 2011; 17:589–604. [PubMed: 21747128]
4. Hayashi K, Matsuda Y, Kawamichi Y, Shiozaki A, Saito S. Smoking during pregnancy increases risks of various obstetric complications: a case-cohort study of the Japan Perinatal Registry Network database. *J Epidemiol*. 2011; 21:61–66. [PubMed: 21088370]
5. Bouhours-Nouet N, May-Panloup P, Coutant R, de Casson FB, Descamps P, et al. Maternal smoking is associated with mitochondrial DNA depletion and respiratory chain complex III deficiency in placenta. *Am J Physiol Endocrinol Metab*. 2005; 288:E171–177. [PubMed: 15585597]
6. Lightowlers RN, Chinnery PF, Turnbull DM, Howell N. Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. *Trends Genet*. 1997; 13:450–455. [PubMed: 9385842]
7. Lee HC, Lu CY, Fahn HJ, Wei YH. Aging- and smoking-associated alteration in the relative content of mitochondrial DNA in human lung. *FEBS Lett*. 1998; 441:292–296. [PubMed: 9883902]
8. Kang D, Hamasaki N. Maintenance of mitochondrial DNA integrity: repair and degradation. *Curr Genet*. 2002; 41:311–322. [PubMed: 12185497]
9. Fliss MS, Usadel H, Caballero OL, Wu L, Buta MR, et al. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. *Science*. 2000; 287:2017. [PubMed: 10720328]
10. Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. *J Biol Chem*. 1997; 272:25409–25412. [PubMed: 9325246]

11. Polyak K, Li Y, Zhu H, Lengauer C, Willson JK, et al. Somatic mutations of the mitochondrial genome in human colorectal tumours. *Nat Genet.* 1998; 20:291–293. [PubMed: 9806551]
12. Barrientos A, Casademont J, Cardellach F, Estivill X, Urbano-Marquez A, et al. Reduced steady-state levels of mitochondrial RNA and increased mitochondrial DNA amount in human brain with aging. *Brain Res Mol Brain Res.* 1997; 52:284–289. [PubMed: 9495550]
13. Fukagawa NK, Li M, Liang P, Russell JC, Sobel BE, et al. Aging and high concentrations of glucose potentiate injury to mitochondrial DNA. *Free Radic Biol Med.* 1999; 27:1437–1443. [PubMed: 10641738]
14. Lezza AM, Pesce V, Cormio A, Fracasso F, Vecchiet J, et al. Increased expression of mitochondrial transcription factor A and nuclear respiratory factor-1 in skeletal muscle from aged human subjects. *FEBS Lett.* 2001; 501:74–78. [PubMed: 11457459]
15. Miro O, Alonso JR, Jarreta D, Casademont J, Urbano-Marquez A, et al. Smoking disturbs mitochondrial respiratory chain function and enhances lipid peroxidation on human circulating lymphocytes. *Carcinogenesis.* 1999; 20:1331–1336. [PubMed: 10383908]
16. Smith PR, Cooper JM, Govan GG, Harding AE, Schapira AH. Smoking and mitochondrial function: a model for environmental toxins. *Q J Med.* 1993; 86:657–660. [PubMed: 8255963]
17. Aoshiba K, Tamaoki J, Nagai A. Acute cigarette smoke exposure induces apoptosis of alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol.* 2001; 281:L1392–1401. [PubMed: 11704535]
18. Lindstrom K, Lindblad F, Hjern A. Preterm birth and attention-deficit/hyperactivity disorder in schoolchildren. *Pediatrics.* 2011; 127:858–865. [PubMed: 21502231]
19. Vogt H, Lindstrom K, Braback L, Hjern A. Preterm birth and inhaled corticosteroid use in 6- to 19-year-olds: a Swedish national cohort study. *Pediatrics.* 2011; 127:1052–1059. [PubMed: 21555500]
20. Ruth CA, Roos N, Hildes-Ripstein E, Brownell M. The influence of gestational age and socioeconomic status on neonatal outcomes in late preterm and early term gestation: A population based study. *BMC Pregnancy Childbirth.* 2012; 12:62. [PubMed: 22748037]
21. Wang X, Tager IB, Van Vunakis H, Speizer FE, Hanrahan JP. Maternal smoking during pregnancy, urine cotinine concentrations, and birth outcomes. A prospective cohort study. *Int J Epidemiol.* 1997; 26:978–988. [PubMed: 9363518]
22. Kramer MS, Seguin L, Lydon J, Goulet L. Socio-economic disparities in pregnancy outcome: why do the poor fare so poorly? *Paediatr Perinat Epidemiol.* 2000; 14:194–210. [PubMed: 10949211]
23. Windham GC, Hopkins B, Fenster L, Swan SH. Prenatal active or passive tobacco smoke exposure and the risk of preterm delivery or low birth weight. *Epidemiology.* 2000; 11:427–433. [PubMed: 10874550]
24. Kramer MS, Platt R, Yang H, McNamara H, Usher RH. Are all growth-restricted newborns created equal(ly)? *Pediatrics.* 1999; 103:599–602. [PubMed: 10049963]
25. Aagaard-Tillery KM, Porter TF, Lane RH, Varner MW, Lacoursiere DY. In utero tobacco exposure is associated with modified effects of maternal factors on fetal growth. *Am J Obstet Gynecol.* 2008; 198:66, e61–66. [PubMed: 18166310]
26. Lattuada D, Colleoni F, Martinelli A, Garretto A, Magni R, et al. Higher mitochondrial DNA content in human IUGR placenta. *Placenta.* 2008; 29:1029–1033. [PubMed: 19007984]
27. Colleoni F, Lattuada D, Garretto A, Massari M, Mando C, et al. Maternal blood mitochondrial DNA content during normal and intrauterine growth restricted (IUGR) pregnancy. *Am J Obstet Gynecol.* 2010; 203:365, e361–366. [PubMed: 20619387]
28. Florescu A, Ferrence R, Einarson T, Selby P, Soldin O, et al. Methods for quantification of exposure to cigarette smoking and environmental tobacco smoke: focus on developmental toxicology. *Ther Drug Monit.* 2009; 31:14–30. [PubMed: 19125149]
29. Mattes W, Yang X, Orr MS, Richter P, Mendrick DL. Biomarkers of tobacco smoke exposure. *Adv Clin Chem.* 2014; 67:1–45. [PubMed: 25735858]
30. Ingvarsson RF, Bjarnason AO, Dagbjartsson A, Hardardottir H, Haraldsson A, et al. The effects of smoking in pregnancy on factors influencing fetal growth. *Acta Paediatr.* 2007; 96:383–386. [PubMed: 17407461]

31. Pringle PJ, Geary MP, Rodeck CH, Kingdom JC, Kayamba-Kay's S, et al. The influence of cigarette smoking on antenatal growth, birth size, and the insulin-like growth factor axis. *J Clin Endocrinol Metab.* 2005; 90:2556–2562. [PubMed: 15713720]
32. Apelberg BJ, Witter FR, Herbstman JB, Calafat AM, Halden RU, et al. Cord serum concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in relation to weight and size at birth. *Environ Health Perspect.* 2007; 115:1670–1676. [PubMed: 18008002]
33. Herbstman JB, Sjodin A, Apelberg BJ, Witter FR, Patterson DG, et al. Determinants of prenatal exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in an urban population. *Environ Health Perspect.* 2007; 115:1794–1800. [PubMed: 18087602]
34. Herbstman JB, Sjodin A, Apelberg BJ, Witter FR, Halden RU, et al. Birth delivery mode modifies the associations between prenatal polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) and neonatal thyroid hormone levels. *Environ Health Perspect.* 2008; 116:1376–1382. [PubMed: 18941581]
35. Wells EM, Navas-Acien A, Herbstman JB, Apelberg BJ, Silbergeld EK, et al. Low-level lead exposure and elevations in blood pressure during pregnancy. *Environ Health Perspect.* 2011; 119:664–669. [PubMed: 21292600]
36. Bernert JT Jr, Turner WE, Pirkle JL, Sosnoff CS, Akins JR, et al. Development and validation of sensitive method for determination of serum cotinine in smokers and nonsmokers by liquid chromatography/atmospheric pressure ionization tandem mass spectrometry. *Clin Chem.* 1997; 43:2281–2291. [PubMed: 9439445]
37. Hoque MO, Lee CC, Cairns P, Schoenberg M, Sidransky D. Genome-wide genetic characterization of bladder cancer: a comparison of high-density single-nucleotide polymorphism arrays and PCR-based microsatellite analysis. *Cancer Res.* 2003; 63:2216–2222. [PubMed: 12727842]
38. Jiang WW, Rosenbaum E, Mambo E, Zahurak M, Masayeva B, et al. Decreased mitochondrial DNA content in posttreatment salivary rinses from head and neck cancer patients. *Clin Cancer Res.* 2006; 12:1564–1569. [PubMed: 16533782]
39. Jiang WW, Masayeva B, Zahurak M, Carvalho AL, Rosenbaum E, et al. Increased mitochondrial DNA content in saliva associated with head and neck cancer. *Clin Cancer Res.* 2005; 11:2486–2491. [PubMed: 15814624]
40. Tu YK. Commentary: Is structural equation modelling a step forward for epidemiologists? *Int J Epidemiol.* 2009; 38:549–551. [PubMed: 19153137]
41. Beran TN, Violato C. Structural equation modeling in medical research: a primer. *BMC Res Notes.* 2010; 3:267. [PubMed: 20969789]
42. Li R, Tsaih SW, Shockley K, Stylianou IM, Wergedal J, et al. Structural model analysis of multiple quantitative traits. *PLoS Genet.* 2006; 2:e114. [PubMed: 16848643]
43. Dyson RM, Palliser HK, Latter JL, Kelly MA, Chwatko G, et al. Interactions of the gasotransmitters contribute to microvascular tone (dys)regulation in the preterm neonate. *PLoS One.* 2015; 10:e0121621. [PubMed: 25807236]
44. van der Linden SL, Leiserowitz AA, Feinberg GD, Maibach EW. The scientific consensus on climate change as a gateway belief: experimental evidence. *PLoS One.* 2015; 10:e0118489. [PubMed: 25714347]
45. StataCorp. Stata Statistical Software: Release 13. College Station, TX: Statacorp LP; 2013.
46. Burke H, Leonardi-Bee J, Hashim A, Pine-Abata H, Chen Y, et al. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics.* 2012; 129:735–744. [PubMed: 22430451]
47. Castles A, Adams EK, Melvin CL, Kelsch C, Boulton ML. Effects of smoking during pregnancy. Five meta-analyses. *Am J Prev Med.* 1999; 16:208–215. [PubMed: 10198660]
48. Jedrychowski WA, Perera FP, Spengler JD, Mroz E, Stigter L, et al. Intrauterine exposure to fine particulate matter as a risk factor for increased susceptibility to acute broncho-pulmonary infections in early childhood. *Int J Hyg Environ Health.* 2013; 216:395–401. [PubMed: 23333083]
49. Keohavong P, Xi L, Day RD, Zhang L, Grant SG, et al. HPRT gene alterations in umbilical cord blood T-lymphocytes in newborns of mothers exposed to tobacco smoke during pregnancy. *Mutat Res.* 2005; 572:156–166. [PubMed: 15790499]

50. Kilic S, Yuksel B, Lortlar N, Sertyel S, Aksu T, et al. Environmental tobacco smoke exposure during intrauterine period promotes granulosa cell apoptosis: a prospective, randomized study. *J Matern Fetal Neonatal Med.* 2012; 25:1904–1908. [PubMed: 22468922]
51. Lee SL, Lam TH, Leung TH, Wong WH, Schooling M, et al. Foetal exposure to maternal passive smoking is associated with childhood asthma, allergic rhinitis, and eczema. *ScientificWorldJournal.* 2012; 2012:542983. [PubMed: 22927783]
52. Llaquet H, Pichini S, Joya X, Papaseit E, Vall O, et al. Biological matrices for the evaluation of exposure to environmental tobacco smoke during prenatal life and childhood. *Anal Bioanal Chem.* 2010; 396:379–399. [PubMed: 19466395]
53. Zalacain M, Sierrasesumaga L, Larrannaga C, Patinno-Garcia A. Effects of benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) in vitro and of maternal smoking in vivo on micronuclei frequencies in fetal cord blood. *Pediatr Res.* 2006; 60:180–184. [PubMed: 16864700]
54. Barrington-Trimis JL, Searles Nielsen S, Preston-Martin S, Gauderman WJ, Holly EA, et al. Parental smoking and risk of childhood brain tumors by functional polymorphisms in polycyclic aromatic hydrocarbon metabolism genes. *PLoS One.* 2013; 8:e79110. [PubMed: 24260161]
55. Bhattacharya S, Beasley M, Pang D, Macfarlane GJ. Maternal and perinatal risk factors for childhood cancer: record linkage study. *BMJ Open.* 2014; 4:e003656.
56. Bocskay KA, Tang D, Orjuela MA, Liu X, Warburton DP, et al. Chromosomal aberrations in cord blood are associated with prenatal exposure to carcinogenic polycyclic aromatic hydrocarbons. *Cancer Epidemiol Biomarkers Prev.* 2005; 14:506–511. [PubMed: 15734979]
57. Brooks DR, Mucci LA, Hatch EE, Cnattingius S. Maternal smoking during pregnancy and risk of brain tumors in the offspring. A prospective study of 1.4 million Swedish births. *Cancer Causes Control.* 2004; 15:997–1005. [PubMed: 15801484]
58. Milne E, Greenop KR, Scott RJ, Ashton LJ, Cohn RJ, et al. Parental smoking and risk of childhood brain tumors. *Int J Cancer.* 2013; 133:253–259. [PubMed: 23280760]
59. Ortega-Garcia JA, Martin M, Lopez-Fernandez MT, Fuster-Soler JL, Donat-Colomer J, et al. Transgenerational tobacco smoke exposure and childhood cancer: an observational study. *J Paediatr Child Health.* 2010; 46:291–295. [PubMed: 20412413]
60. Ng SP, Silverstone AE, Lai ZW, Zelikoff JT. Effects of prenatal exposure to cigarette smoke on offspring tumor susceptibility and associated immune mechanisms. *Toxicol Sci.* 2006; 89:135–144. [PubMed: 16207940]
61. Marcelino LA, Thilly WG. Mitochondrial mutagenesis in human cells and tissues. *Mutat Res.* 1999; 434:177–203. [PubMed: 10486591]
62. Penta JS, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. *Mutat Res.* 2001; 488:119–133. [PubMed: 11344040]
63. Armani C, Landini L Jr, Leone A. Molecular and biochemical changes of the cardiovascular system due to smoking exposure. *Curr Pharm Des.* 2009; 15:1038–1053. [PubMed: 19355946]
64. Banzet N, Francois D, Polla BS. Tobacco smoke induces mitochondrial depolarization along with cell death: effects of antioxidants. *Redox Rep.* 1999; 4:229–236. [PubMed: 10731097]
65. Smith CJ, Perfetti TA, Garg R, Hansch C. IARC carcinogens reported in cigarette mainstream smoke and their calculated log P values. *Food Chem Toxicol.* 2003; 41:807–817. [PubMed: 12738186]
66. Schwarz KB, Cox J, Sharma S, Witter F, Clement L, et al. Cigarette smoking is pro-oxidant in pregnant women regardless of antioxidant nutrient intake. *Journal of Nutritional and Environmental Medicine.* 1995; 5:225–234.
67. Longo LD. Carbon monoxide: effects on oxygenation of the fetus in utero. *Science.* 1976; 194:523–525. [PubMed: 973133]
68. Suzuki K, Horiguchi T, Comas-Urrutia AC, Mueller-Heubach E, Morishima HO, et al. Pharmacologic effects of nicotine upon the fetus and mother in the rhesus monkey. *Am J Obstet Gynecol.* 1971; 111:1092–1101. [PubMed: 5001981]
69. Lehtovirta P, Forss M. The acute effect of smoking on intervillous blood flow of the placenta. *Br J Obstet Gynaecol.* 1978; 85:729–731. [PubMed: 708655]

70. Pettigrew AR, Logan RW, Willocks J. Smoking in pregnancy--effects on birth weight and on cyanide and thiocyanate levels in mother and baby. *Br J Obstet Gynaecol.* 1977; 84:31–34. [PubMed: 843468]
71. Wakefield SL, Lane M, Mitchell M. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. *Biol Reprod.* 2011; 84:572–580. [PubMed: 21076083]
72. Muralimanoharan S, Maloyan A, Mele J, Guo C, Myatt LG, et al. MIR-210 modulates mitochondrial respiration in placenta with preeclampsia. *Placenta.* 2012; 33:816–823. [PubMed: 22840297]
73. Morten K, Field P, Ashley N, Williams KA, Harris D, et al. Fetal and neonatal exposure to AZT and low-protein diet affects glucose homeostasis: a model with implications for AIDS prevention. *Am J Physiol Endocrinol Metab.* 2005; 289:E1115–1118. [PubMed: 16014351]
74. Poidatz D, Dos Santos E, Duval F, Moindjie H, Serazin V, et al. Involvement of estrogen-related receptor-gamma and mitochondrial content in intrauterine growth restriction and preeclampsia. *Fertil Steril.* 2015
75. Chiaratti MR, Malik S, Diot A, Rapa E, Macleod L, et al. Is Placental Mitochondrial Function a Regulator that Matches Fetal and Placental Growth to Maternal Nutrient Intake in the Mouse? *PLoS One.* 2015; 10:e0130631. [PubMed: 26132581]
76. Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, et al. Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. *Am J Physiol Heart Circ Physiol.* 2013; 305:H459–476. [PubMed: 23748424]
77. MITOMAP. Mitochondrial DNA Map b&w). A Human Mitochondrial Genome Database. 2013. <http://www.mitomap.org>

Fig. 1.
MT-CO1 and *MT-CO2* levels in umbilical cord and umbilical cord serum from newborns exposed to very low levels of cotinine in utero – Non-Smokers (n=106, geometric mean = 0.05 ng/ml); and babies exposed to cotinine in utero – Active and Passive Smokers (n=36, mean = 12.1 ng/ml).

Fig. 2.

Structural Equation Modeling results for a) *MT-CO1* and b) *MT-CO2*. Figures show relationships between *MT-CO1* and *MT-CO2*, with smoking (natural log of cotinine, ng/ml), *IGF-1* (insulin-like growth factor-1), gestational hypertension (HTN), and birth outcomes -- gestational age (days), and head circumference (cm). Other variables controlled in final models were maternal prepregnancy body mass index (BMI) (obese), race (African American) and sex (male). Diagrams illustrate standardized coefficients for each direct pathway, error terms, and standard deviations (in variable boxes).

Umbilical cord serum *MT-CO1*, *MT-CO2* and cotinine levels (ng/ml, natural log) for population subgroups, geometric means and 95% confidence levels.

Table 1a

Sex	<i>MT-CO1</i>			<i>MT-CO2</i>			Cotinine		
	GM	95% CI	* p	GM	95% CI	* p	GM	95% CI	* p
Male (n=78)	76.6	(66.7, 87.9)		73.5	(64.5, 83.8)		0.16	(0.08, 0.31)	
Female (n=64)	55.2	(45.5, 67.0)	0.006	54.8	(45.9, 65.5)	0.008	0.27	(0.13, 0.56)	NS
Maternal Race									
Caucasian (n=30)	72.4	(57.2, 91.6)	NS	62.7	(50.2, 78.5)	NS	0.04	(0.01, 0.12)	
Asian (n=9)	58.3	(39.6, 86.0)		60.9	(44.4, 83.4)		0.02	(0.01, 0.03)	NS
African American (n=103)	65.0	(56.2, 75.2)	NS	65.2	(56.9, 74.7)	NS	0.40	(0.23, 0.69)	0.0002
Maternal Age									
<18 (n=12)	59.4	(41.3, 85.5)	NS	56.2	(39.4, 80.1)	NS	0.86	(0.18, 4.27)	0.10
18–35 (n=119)	66.5	(58.4, 75.7)		65.5	(58.1, 73.8)		0.20	(0.12, 0.34)	
35+ (n=11)	72.9	(44.7, 119.0)	NS	65.5	(40.9, 104.9)	NS	0.05	(0.005, 0.23)	0.12
Prepregnancy BMI									
Underweight (n=9)	79.2	(50.1, 125.2)	NS	79.0	(48.5, 128.7)	NS	0.11	(0.01, 1.04)	NS
Normal (n=60)	72.5	(62.7, 83.7)		69.4	(60.7, 79.4)		0.14	(0.06, 0.29)	
Overweight (n=33)	66.6	(48.2, 92.1)	NS	65.5	(48.6, 88.3)	NS	0.29	(0.10, 0.85)	NS
Obese (n=36)	55.4	(44.6, 68.9)	0.06	54.2	(44.3, 66.3)	0.07	0.31	(0.11, 0.86)	0.2
Smoking**									
Nonsmoker (n=106)	61.0	(53.3, 69.8)		59.0	(52.1, 66.8)		0.05	(0.04, 0.07)	
Passive Smoker (n=11)	83.3	(54.5, 127.2)	0.16	76.6	(51.6, 113.9)	0.2	1.78	(1.30, 2.44)	<0.0001
Active Smoker (n=25)	83.7	(62.4, 112.1)	0.04	86.5	(65.7, 114.0)	0.008	28.1	(11.9, 66.5)	<0.0001
GHTN									
No (n=130)	68.4	(60.8, 77.0)		66.0	(59.0, 73.7)		0.20	(0.12, 0.33)	
Yes (n=12)	44.9	(25.7, 78.4)	0.05	49.4	(29.7, 82.1)	0.18	0.25	(0.02, 0.33)	NS
Diabetes									
No (n=126)	66.1	(58.1, 75.2)		64.5	(57.2, 72.7)		0.24	(0.14, 0.41)	
Yes (n=16)	65.7	(51.7, 83.6)	NS	63.9	(50.2, 81.4)	NS	0.05	(0.01, 0.19)	NS

	<i>MT-COI</i>			<i>MT-CO2</i>			Cotinine		
	GM	95% CI	p *	GM	95% CI	p *	GM	95% CI	p *
Sex									
Prior Pregnancies (none)									
No (n=59)	58.4	(50.3, 67.7)		55.9	(49.0, 63.8)		0.12	(0.06, 0.23)	
Yes (n=83)	72.7	(61.4, 86.1)	0.07	71.7	(61.2, 84.0)	0.02	0.29	(0.14, 0.59)	0.08
Medical Coverage									
Public (n=44)	58.6	(46.7, 73.6)	NS	57.5	(46.6, 70.9)	NS	0.55	(0.24, 1.29)	<0.0001
Private (n=54)	68.1	(57.2, 81.1)		65.5	(55.7, 76.9)		0.05	(0.02, 0.09)	
Missing (n=44)	72.7	(58.2, 90.7)	NS	71.6	(58.1, 88.2)	NS	0.44	(0.17, 1.11)	<0.0001

* P-values by T- test.

**

As determined by cord serum cotinine levels at birth. Mothers who reported smoking during pregnancy with umbilical cord serum cotinine levels below cutoffs were classified as smokers.

GHTN - Gestational hypertension

Pairwise correlations birth outcomes (gestational age, birthweight, head circumference, length); maternal variables (age, body mass index); and serum biomarkers (cotinine, *IGF-I*, *MT-COI* and *MT-CO2*), correlation coefficients and p-values. Baltimore THREE Study.

Table 1b

	GestAge (d)	Birthweight (g)	HeadCirc (cm)	Length(cm)	MatAge (y)	BMI(kg/m2)	Cotinineug/ml(ln)	IGF-Iug/ml(ln)	MT-COI(ln)
Birth weight (g) p-value	0.65 <0.0001								
Head Circ (cm) p-value	0.51 <0.0001	0.66 <0.0001							
Length (cm) p-value	0.58 <0.0001	0.79 <0.0001	0.50 <0.0001						
Maternal Age (y) p-value	-0.11 0.21	-0.05 0.57	0.08 0.34	0.03 0.73					
BMI (kg/m2) p-value	-0.01 0.90	0.01 0.90	-0.05 0.55	0.10 0.22	0.01 0.87				
Cotinine ng/ml (ln) p-value	-0.17 <0.05	-0.23 <0.01	-0.24 <0.01	-0.17 <0.05	-0.26 <0.05	0.12 0.17			
<i>IGF-I</i> ng/ml (ln) p-value	0.17 <0.05	0.43 <0.0001	0.28 <0.001	0.28 <0.001	0.00 0.99	-0.03 0.71	-0.23 <0.01		
<i>MT-COI</i> p-value	-0.18 <0.05	-0.06 0.47	0.02 0.85	-0.10 0.24	0.08 0.32	-0.07 0.44	0.21 0.01	0.04 0.66	
<i>MT-CO2</i> p-value	-0.23 <0.01	-0.10 0.22	0.02 0.81	-0.14 0.09	0.08 0.36	-0.08 0.35	0.25 <0.01	0.00 0.99	0.95 <0.00011

Associations between demographic factors, maternal factors and umbilical cord serum *IGF-I*, *MT-COI*, *MT-CO2* and cotinine levels with gestational age, birthweight, length and head circumference by bivariate linear regression analysis. Baltimore THREE Study.

Table 1c

Dependent Variables	Gestational Age (d)		Birthweight* (gm)		Length* (cm)		Head Circumference* (cm)	
	Beta	95% CI	Beta	95% CI	Beta	95% CI	Beta	95% CI
Independent Variables								
Gestational Age (days)	X	X	25	(21,29)	0.11	(0.09,0.14)	0.06	(0.05,0.08)
Maternal Age (y)								
<18	-5.8	(-14.2, 2.6)	100	(-306, 108)	0.1	(-0.4, 2.3)	0.3	(-0.6, 1.2)
>35	-5.1	(-13.9, 3.6)	173	(-100, 446)	1.2	(-0.2, 2.7)	0.5	(-0.5, 1.5)
Race (African American)	-4.8	(-10.0, 0.3)	28	(-137, 194)	0.9	(-0.8, 1.0)	-0.1	(-0.7, 0.4)
Sex (Male)	0.0	(-4.7, 4.7)	-179	(-322, -34)	-1.0	(-1.7, -0.3)	-0.4	(-0.9, 0.1)
Prepregnancy BMI (kg/m ²)								
Underweight	-9.6	(-19.2, 0.1)	-304	(-613, 4)	-1.8	(-3.4, -0.2)	-0.8	(-1.9, 0.3)
Overweight	-0.4	(-6.2, 5.5)	-70	(-254, 114)	-0.3	(-1.3, 0.6)	-0.7	(-1.4, 10.1)
Obese	-2.3	(-7.9, 3.4)	-50	(-230, 129)	0.3	(-0.6, 1.2)	-0.5	(-1.2, 0.1)
GHTN	-11.4	(-19.6, -3.2)	-66	(-336, 203)	0.3	(-1.1, 1.7)	-0.3	(-1.2, 0.6)
Prior pregnancies (Yes)	-2.1	(-6.9, 2.6)	-141	(-288, 6)	-0.4	(-1.2, 0.4)	-0.6	(-1.1, -0.1)
Private insurance (Yes)	3.9	(-0.5, 8.2)	18	(-171, 208)	0.3	(-0.6, 1.2)	0.3	(-0.9, 0.4)
Diabetes (Yes, all)	-2.2	(-9.7, 5.2)	191	(-38, 421)	0.5	(-0.6, 1.7)	1.4	(0.6, 2.2)
Insulin-like Growth Factor 1 (<i>IGF-I</i>) (natural log)	5.0	(0.1, 9.9)	391	(248, 535)	1.2	(0.4, 2.0)	0.8	(0.3, 1.4)
Cotinine (natural log, ng/ml)	-0.8	(-1.6, -0.03)	-23	(-48, 2)	-0.1	(-0.2, 0.03)	-0.1	(-0.2, -0.03)
<i>MT-COI</i>	-0.04	(-0.08, -0.004)	0.5	(-0.6, 1.7)	0.001	(-0.005, 0.007)	0.003	(-0.001, 0.007)
<i>MT-CO2</i>	-0.06	(-0.10, -0.02)	0.5	(0.8, 1.8)	-0.0003	(-0.007, 0.006)	0.004	(0.0001, 0.009)

* All models (except model for gestational age) control for gestational age.

Table 2

Interrelationships between umbilical cord serum cotinine (ng/ml, ln), *MT-CO* and *IGF-I** (ng/ml, ln) levels and associations with gestational age (d) and head circumference (cm) by structural equation modeling on the natural log of *MT-CO1* and *MT-CO2*, Baltimore THREE Study.

Relationship	<i>MT-CO1</i>			<i>MT-CO2</i>		
	beta	95% Confidence Interval	p-value	beta	95% Confidence Interval	p-value
ln (Cotinine) --> ln (<i>IGF-I</i>)	-0.04	-0.07 -0.01	0.003	-0.04	-0.07 -0.01	0.003
ln (Cotinine) --> <i>MT-CO</i>	0.06	0.02 0.09	0.006	0.06	0.03 0.1	<0.001
ln (Cotinine) --> Gestational Age (days)	-0.9	-1.7 -0.1	0.02	-0.9	-1.7 -0.1	0.02
ln (Cotinine) --> Head Circumference (cm)	-0.2	-0.3 -0.07	0.003	-0.2	-0.3 -0.07	0.001
ln (<i>IGF-I</i>) --> Head Circumference (cm)	0.7	0.2 1.2	0.009	0.7	0.2 1.2	0.01
<i>MT-CO</i> --> Gestational Age	-3.0	-6.0 0.0	0.05	-3.7	-7.0 -0.4	0.03
<i>MT-CO</i> --> Head Circumference	0.2	-0.2 0.6	0.08	0.3	-0.2 0.7	0.09
Model Fit	<i>MT-CO1</i>			<i>MT-CO2</i>		
Likelihood Ratio of Model vs. Saturated	19.96			18.43		
Probability > Chi Square	0.28			0.43		

* *IGF-I* = Intrauterine Growth Factor

Both sets of models control for: maternal BMI (obese), race (African American) and sex as well as possible mediation by gestational hypertension.