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Association between mitochondrial DNA copy number and neurodevelopmental outcomes among black and white preterm infants up to two years of age

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Abstract

Objectives: Mitochondrial DNA copy number (mtDNAcn) is associated with mitochondrial function, with abnormal copy numbers having been linked to various disease states. Our study aims to understand the association between infant mtDNAcn and infant neurodevelopment, as well as the association with racial disparities.

Methods: A longitudinal study was conducted with 55 preterm infants from whom a single blood sample was collected during their Neonatal intensive care unit (NICU) stay and used to analyze mtDNAcn. In addition, the NICU Network Neurobehavioral Scale at 36–38 postmenstrual age (PMA) and the Bayley Scale of Infant and Toddler Development (Bayley) Edition III at 1 and 2 years of corrected age were both conducted. Linear regression models were performed to investigate the relationship between infant clinical characteristics, infant neurobehavioral outcomes, and mtDNAcn.

Results: The majority of infants studied were white (72.73%), non-Hispanic (70.91%), males (54.55%), delivered through C-section (72.73%), and without preterm premature rupture of membrane (76.36%). Increased mtDNAcn was associated with younger birth gestational age (< 30.57 wk, $P < 0.001$). In addition, the opposite associations between mtDNAcn and neurodevelopmental outcomes were observed between white and black infants up to 1 year of gestational age.

Conclusions: Increased mtDNAcn in white infants, and decreased mtDNAcn in black infants may be considered significant predictors of poor early-life neurodevelopmental outcomes in infants. A better understanding of the underlying mechanisms contributing to infant disparity in mtDNAcn and how low or high copy number impacts infant outcomes is essential.

Keywords: Preterm infants, Mitochondrial DNA copy number (mtDNAcn), Racial disparity, PPROM, Neurodevelopmental outcomes

Introduction

Mitochondria contain their own haploid, circular genome which in humans encodes 37 genes, including 13 proteins of the Oxidative Phosphorylation system[1], critical for the production of adenosine triphosphate (ATP). Because more than 95% of mitochondrial proteins are encoded in nuclear DNA (nDNA), understanding proper mitochondrial physiological functioning

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requires the analysis of genetic information encoded by both genomes[2]. Nucleated cells contain a single diploid genome copy where maternal and paternal information is coded[3]. By comparison, nucleated cells contain a variable number of mitochondrial DNA (mtDNA), from 100 to 10,000 copies depending on cell type, which are exclusively maternally inherited[4]. Mitochondrial DNA copy number (mtDNAcn) is typically quantified as the number of mtDNA copies normalized by the number of nDNA copy number (nDNA) copies and has long been used as a metric of mitochondrial health[5].

Studies show that there is not a simple correlation between mtDNAcn and mitochondrial health, as both decreases and increases in mtDNAcn have been associated with heritable and acquired mitochondrial dysfunction[6]. For example, mtDNAcn depletion is related to lower mitochondrial energy generation,[7] whereas increased mtDNAcn is related to compensatory upregulation of the Oxidative Phosphorylation system[8]. This system, which links the tricarboxylic acid cycle to the production of ATP, is upregulated in response to compromised mitochondrial function[8]. Thus, both lower and higher variability in mtDNAcn have been related to multiple diseases including diabetes, cancer, neurological disorders, and cardiovascular diseases[9–11]. mtDNAcn varies not only with specific measures of mitochondrial function but also with environmental factors as well as between races[12]. In addition, lower mtDNAcn levels have been associated with an increased risk of chronic disease, including an increased risk of diabetes among white adults[13]. Furthermore, lower mtDNAcn levels were found in female black adults to be associated with risk of frailty[14]. This pattern of lower mtDNAcn was also notable among men compared with women and among black adults who reported having smoked heavily during their life[14,15]. However, the author did not look at the difference based on the race. In addition, mtDNAcn was negatively associated with age and positively with adverse experiences during early life[16]. However, the impact of mtDNAcn on infants' neurodevelopmental outcomes related to racial disparity remains unclear.

Thus, this study aims to explore the associations between blood mtDNAcn, infant early life experience during Neonatal intensive care unit (NICU) hospitalization, and infant neurodevelopmental outcomes among black and white preterm infants. To this end, we used a well-validated droplet digital Polymerase chain reaction (PCR) method for assessing mtDNAcn using genome DNA extracted from blood samples[5]. Our hypothesis is that variations in mtDNAcn can serve as biomarkers to predict the influence of early-life adverse experiences and racial disparities on neurodevelopmental outcomes in preterm infants.

Methods

Experimental design

A longitudinal study design was used to investigate the associations between infant clinical and medical characteristics, blood mtDNAcn, and infant neurodevelopmental outcomes. This study was approved by the Institutional Review Board at 2 affiliated level III/IV NICUs in Connecticut.

Subjects

Medically stable preterm infants recruited between September 2017 and July 2022 at 2 NICU sites and who met the inclusion criteria were invited to participate in the study. Inclusion criteria were: (1) preterm infants between 28 weeks and 32 weeks gestational age (GA) at birth and (2) consent from parents with minimal age requirement ($\geq$ 18 y). Exclusion criteria included infants who: (1) had a known congenital or chromosomal abnormality, (2) experienced severe periventricular/intraventricular hemorrhage ($\geq$ grade III), (3) had undergone surgery, and/or (4) were exposed to an illicit substance during pregnancy.

Clinical and medical characteristics

Preterm infants' clinical characteristics were collected by NICU clinic research nurses and included demographics, delivery method, history of preterm premature rupture of membrane (PPROM), race, birth GA, birth weight, birth length, birth head circumference, and the length of hospital stay data.

Neurobehavioral measures at the NICU

Infants' neurobehavioral outcomes were measured using the NICU Network Neurobehavioral Scale (NNNS), which included 115 items and was categorized into 13 summary scores[17]. NNNS scores were assessed when infants reached a postmenstrual age (PMA) of 36–38 weeks. As suggested by previous findings, we focused on several main summary scores in this study including stress/abstinence (nstress), need for handling (nhandle), self-regulation (nregulation), quality of movement (nqmove), attention (nattention), arousal (narousal), reflex (nrefelx), excitability (nexcitability), and lethargy (nlethargy)[18,19].

The nstress summary score measures the signs of neonatal abstinence and stress in high-risk preterm infants. The nhandle summary score measures the amount of effort from the examiner to elicit infants' attention. The nregulation summary score measures infant's ability to follow auditory and visual orientation testing. The nqmove summary score quantifies infant maturity and modulation of the movement of limbs. The nattention score measures infants' responses to visual and auditory stimulation. The narousal score quantifies the overall level of arousal status during the examination. The nrefelex score quantifies the presence and the strength of newborn nonoptimal responses. In addition, the high levels (nexcitability) and low levels (nlethargy) of motor, state, and physiological reactivity of infants were documented[19].

Lower scores on nstress, nhandle, nreflex, nexcitability, and nlethargy subscales, and higher scores on nregulation, nqmove, and nattention subscales indicate better neurobehavioral outcomes, whereas the moderate scores of narousal indicate better response. Specifically, low narousal scores indicate that the infant is difficult to respond to, whereas high narousal scores indicate that the infant is easily upset during the examination.

Neurodevelopmental outcomes at 1 and 2 years of corrected age

Infant neurodevelopment was assessed using the Bayley Scale of Infant and Toddler Development (Bayley) Edition III[20] during follow-up visits when infants reached 8–12 and 18–24 months corrected age (CA)[21]. Bayley scores focused on cognitive, language, and motor composite scores and higher scores reflect better development.

Blood mitochondrial DNA copy number quantification

During NICU stay, at baseline and after receiving parental informed consent, 1 mL of peripheral venous blood was collected from each infant in EDTA tubes (Fisher Scientific, #02-687-107) as part of the routine blood draw for clinical care in the NICU, thus minimizing additional needle sticks to the infant. All blood samples were stored in a freezer at -80°C until genomic DNA (gDNA) extraction, allowing mtDNA and nDNA isolation. Specifically, gDNA was isolated from 100 uL of blood using the DNeasy Blood and Tissue kit from Qiagen (Qiagen) following the manufacturer's recommended protocol. Extracted gDNA samples were checked for purity, concentration, and integrity before preparation for the digital droplet PCR copy number assay. Purity ratios were determined for each sample using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Samples were quantified and normalized using the double-stranded (dsDNA) High Sensitivity Assay for Qubit 3.0 (Life Technologies). To further assess DNA quality, gDNA was analyzed on the Agilent TapeStation 4200 (Agilent Technologies) using the gDNA assay.

To assess mtDNA copy number in these samples, probe assays targeting the mitochondrial genome were obtained from BioRad (BioRad Life Science) for use on the BioRad Digital Droplet QX200 system. Probe assay information is as follows: *MT-ND1* (assay ID: dHsaCPE5029120) with context sequence (CTCTAGCCTAGCCGTTTACTCAATCCTCTGATCAGGGT GAGCATCAAACTCAAACTACGCCCTGATCGGCGCACTG CGAGCAGTAGCCCAAACAATCTCATATGAAGTCACCCT AGCCATCATTCTACT ATCAACATTACTAATAA) and *EIF2C1* (assay ID: dHsaCP1000002) with context sequence (TGGTTCGG CTTTCACCAGTCTGTGCGCCCTGCCATGTGGAAGATGAT GCTCAACATTGATGGTGAGTGGGGAGAGCTATGGAGCCA GGGGCACCCCAAGTCCAGTGACCACACTCCCAGCCTC). gDNA samples were normalized to a standardized concentration of 5 ng/μL and prepared for droplet generation following the manufacturer's recommended protocol for droplet digital PCR Supermix for Probes (No dUTP). Master mix setup followed manufacturer recommendations for 20 μL reaction volumes, using 5 μL of a 1:10 dilution of normalized gDNA and no restriction enzyme. Emulsions were collected from the microfluidic chip, prepared for thermal cycling and then droplets were read by the QX200 droplet reader. Samples were analyzed by the QuantaLife Software to determine the absolute mitochondrial copy number (copies/uL). The mtDNAcn was then calculated by using the absolute copies of mtDNA molecules from *MT-ND1* genes and normalized to *EIF2C1* copy number[5].

Statistical analyses

Data analyses were performed using R (version 4.2.0). The general characteristics of the samples were described using descriptive statistics. Quantitative variables were described using means and SDs, whereas qualitative variables were described using frequencies and percentages. The primary outcomes were the log-transformed mtDNAcn, NNNS subscale scores, and Bayley scores. Linear regression models were performed to investigate the relationships between the primary neurobehavioral outcomes and infant age, sex, race, PPROM, NNNS score, and log-transformed mtDNAcn. Bayesian Information Criterion was used as the selection criteria to select a set of candidate models while holding the control variables (infant age, sex, race, and PPROM) constant across all models. The race-differentiated association between mtDNAcn and NNNS for infants during their NICU stay, and the association between mtDNAcn and Bayley scores for infants' neurodevelopment at 1 and 2 years of age were assessed through unadjusted and adjusted linear regression models. Linear models with interaction terms between mtDNAcn and race were introduced to assess the race-differentiated association between mtDNAcn and NNNS for infants during their NICU stay, and the association between mtDNAcn and Bayley scores for infants' neurodevelopment at ages 1 and 2 years. Comprehensive diagnostics were performed on these candidate models to identify the most parsimonious model that provided the best balance between model fit and complexity. All statistical tests were examined at a 5% level of significance.

Results

Demographic characteristics of preterm infants

The majority of the preterm infants in our study were males (54.55%), white (70.91%), and non-Hispanic (72.73%). The infants were born at 27.97 ± 2.55 weeks of GA with 1055.44 ± 363.74 g birth weight. Most of the study infants had C-section birth (72.73%), had no experience of PPROM (76.36%), and were treated with an antibiotic in the first 3 days after birth

Table 1

Demographic characteristics of preterm infants.

Variables	Total; N (%)
Sex	
Male	30 (54.55)
Female	25 (45.45)
Race	
White	39 (70.91)
Black	13 (23.64)
Other	3 (5.45)
Ethnicity	
Hispanic	15 (27.27)
Non-Hispanic	40 (72.73)
Delivery	
C-section	40 (72.73)
Vaginal	15 (27.27)
PPROM	
Yes	13 (23.64)
No	42 (76.36)
Antibiotic use 3 d pre-birth	
Yes	24 (72.73)
No	7 (21.21)
Unknown	2 (6.06)
Antibiotic use 3 d post-birth	
Yes	6 (18.18)
No	25 (75.76)
Unknown	2 (6.06)
Variables	**Mean (SD)**
Birth GA (wk)	27.97 (2.55)
Birth weight (g)	1055.44 (363.74)
Birth body length (cm)	36.03 (3.70)
Birth HC (cm)	25.09 (2.51)
SNAPEII	27.45 (21.06)
Hospital stays (d)	74.09 (38.16)

GA indicates gestational age; HC, head circumference; PPROM, preterm premature rupture of membrane; SNAPEII, Score for Neonatal Acute Physiology with Perinatal Extension-II.

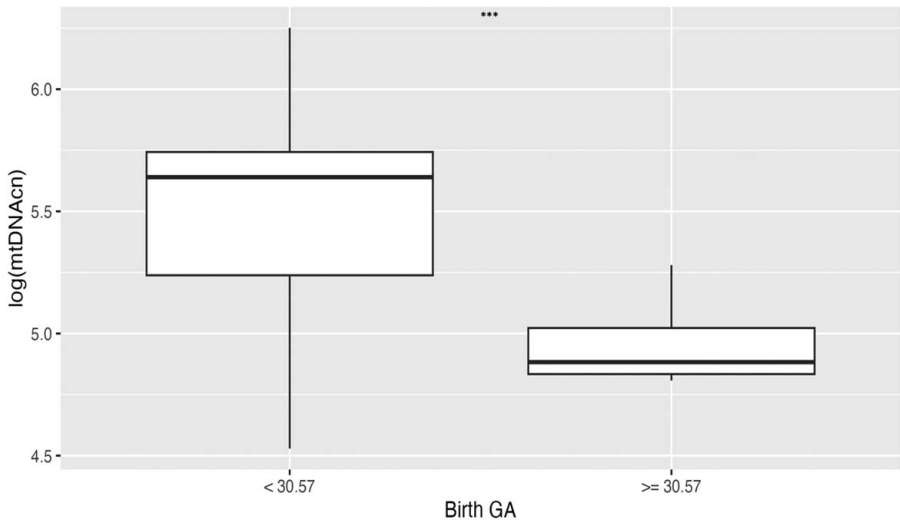

Figure 1. Blood mtDNAcn in preterm infants by GA. Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Blood mtDNAcn were significantly higher in the younger GA group (< 30.57 wk; "***" indicates $P < 0.001$) vs the older GA group (≥ 30.57 wk of GA). GA indicates gestational age; mtDNAcn, mitochondrial DNA copy number.

(72.73%). The average NICU hospitalization was 74.09 ± 38.16 days (Table 1).

Mitochondrial DNA copy number varied with actual gestational age

We previously found the birth GA of 30.57 weeks as a cutoff predicting neurodevelopmental outcomes[18]. In this study, we found that mtDNAcn was significantly higher in the younger GA group (< 30.57 wk; $P < 0.001$; Fig. 1). No significant associations were observed between the level of mtDNAcn and other clinical characteristics, including race.

Association between mitochondrial DNA copy number and neurobehavior at 36–38 postmenstrual age

Negative associations were observed between blood mtDNAcn and nqmove ($P < 0.01$), nattention, and nregulation. Positive associations were observed between blood mtDNAcn and nstress ($P < 0.05$), nhandle, and nrefelex (Fig. 2). There was no significant correlation between mtDNAcn and NNNS

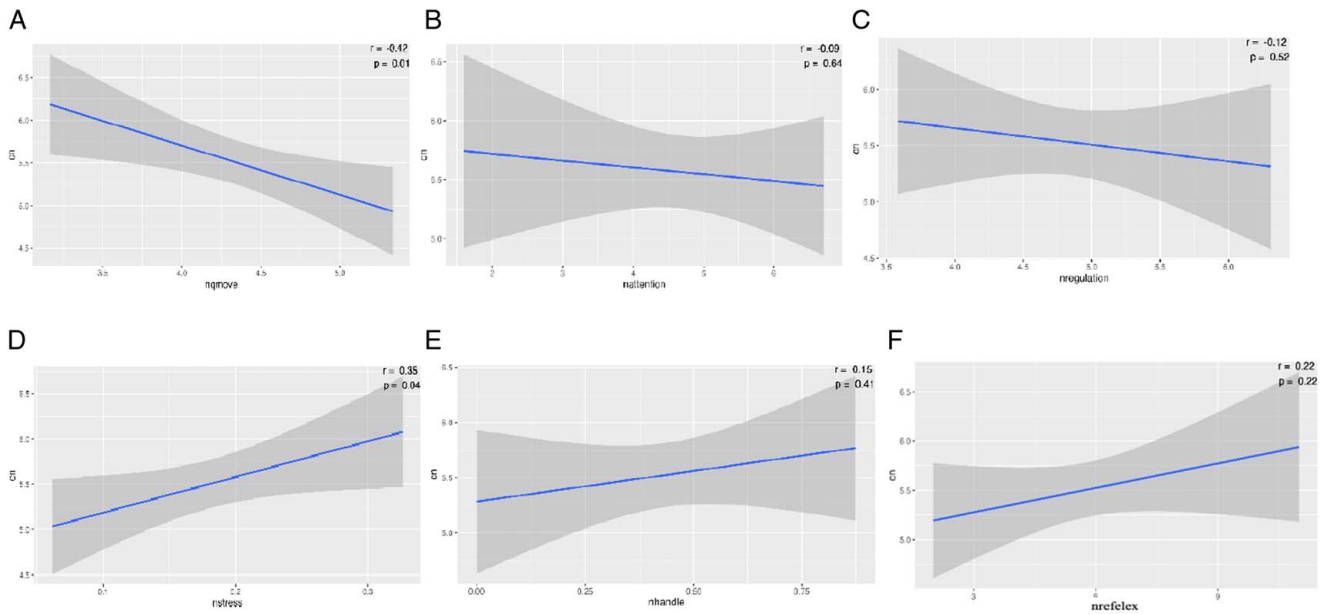

Figure 2. The association between mtDNAcn and neurobehavioral outcomes at 36–38 PMA. The negative associations were observed between mtDNAcn and (A) nqmove ($P < 0.01$), (B) nattention, and (C) nregulation. The positive associations were observed between mtDNAcn and (D) nstress ($P < 0.05$), (E) nhandle, and (F) nrefelex. mtDNAcn indicates mitochondrial DNA copy number; PMA, postmenstrual age.

Table 2

Association between mtDNAcn and neurobehavior at NICU.

	Coefficient		
Variables	nstress	nexcitablity	narousal
mtDNAcn	−0.40	−0.13	0.70
White	−0.10	0.57	−0.09
Male	−0.81	0.52	0.71*
PPROM	0.33*	0.36*	0.31*
Birth GA	0.01	−0.07	0.23

*$P < 0.05$.

GA indicates gestational age; mtDNAcn, mitochondrial DNA copy number; NICU, neonatal intensive care unit; PPROM, preterm premature rupture of membrane.

subscale scores when we adjusted the clinical variables including infants' race, sex, risk of PPROM, and birth GA. However, we observed a positive association between PPROM and NNNS subscales including nstress, nexcitability, and narousal ($P < 0.05$; Table 2).

Association between mitochondrial DNA copy number and neurodevelopment at 1 and 2 years of age

When adjusted for the infant demographic and health characteristics including race, sex, risk of PPROM, and birth GA, at follow-up 8 to 12 months CA, infants' Bayley cognitive composite scores were lower if they had higher blood mtDNAcn ($P < 0.05$). Male infants had lower Bayley cognitive and motor composite scores ($P < 0.05$) compared with female cohorts (Table 3). There were no significant associations between Bayley composite scores and mtDNAcn at 18–24 months of CA follow-up.

Racial differences of association between mitochondrial DNA copy number and neurodevelopmental outcomes

Average scores of each NNNS subscale were computed (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/NR9/A27). At 36–38 weeks of PMA, we observed positive associations between mtDNAcn and nhandle ($P < 0.05$), nstress ($P < 0.05$), and nlethargy ($P < 0.05$). In addition, we observed a negative association between mtDNAcn and narousal ($P < 0.01$) in white infants. Interestingly, these correlations were reversed for black infants (Fig. 3). We also observed the negative association between mtDNAcn and cognitive and language composite scores ($P < 0.05$) at 8–12 months of CA follow-up white infants. However, the positive association between black infants' mtDNAcn and their cognitive and language composite scores ($P < 0.05$) at 8–12 months of CA follow-up was observed in this study (Fig. 4). There were no significant findings for either white or black infants at 18–24 months of CA follow-up.

Table 3

Association between mtDNAcn and development at 1 year of age.

	Coefficient		
Variables	Cognitive	Language	Motor
mtDNAcn	−0.42*	−0.30	−0.31
White	0.44	−0.11	0.41
Male	−0.84*	−0.48	−0.83*
PPROM	−0.18	−0.16	0.80
Birth GA	0.17	0.25	0.17

*$P < 0.05$.

GA indicates gestational age; mtDNAcn, mitochondrial DNA copy number; PPROM, preterm premature rupture of membrane.

Discussion

To our knowledge, this study represented the initial investigation into the correlations between blood mtDNAcn and neurobehavior and neurodevelopment of preterm infants, spanning from their NICU stay to 2 years of CA. Consistent with other studies of an age-dependent decrease in mtDNAcn, we also observed an association between higher blood mtDNAcn and younger birth GA in preterm infants[22]. Although the mechanisms underlying this association were not fully understood, early-life pain/stress in preterm infants could induce reactive oxygen species (ROS) generation in infants, potentially damaging their mitochondrial function[23]. As a compensated response, increased mitochondrial biogenesis, with a corresponding rise in mtDNAcn, might help preserve mitochondrial function and enhance energy production[24]. This adaptation could be particularly crucial to meet the elevated demand for ATP generation during the growth of preterm infants[25].

Our previous separate study found that birth before 30.57 weeks of GA could serve as a cutoff to predict unfavorable neurobehavioral outcomes in preterm infants[18]. Using this cutoff, in this study, we categorized black and white infants into subgroups to examine the mtDNAcn variation across different GA groups. In the current study, we found that white infants born before 30.57 weeks of GA had significantly increased mtDNAcn, suggesting a link between higher mtDNAcn and unfavorable neurobehavior. In contrast, the opposite was observed among black infants. Unlike other studies that reported no racial difference in mitochondrial function[26], our findings are intriguing and suggest different underlying mechanisms by race. Future studies with larger cohorts are warranted to confirm these results.

In addition, our study observed the distinct patterns of mtDNAcn variation associated with neurobehavioral and neurodevelopmental outcomes among black and white preterm infants. For neurobehavioral outcomes, we found that white infants with higher mtDNAcn exhibited more stress and abstinence signs, jittery and startle body movement, and were more easily aroused to fuss and cry at 36–38 weeks of PMA. Conversely, an opposite trend was observed in black infants. This association was also reflected in neurodevelopmental outcomes, including poor cognitive status and language ability during the 1 year of CA follow-up. Although mtDNAcn is an indirect measure of mitochondrial function/dysfunction, the observed link between increased mtDNAcn and a decline in language and cognitive abilities suggests mitochondrial dysfunction related to overproduced ROS, potentially leading to impaired brain cells in infants[26].

Although our study did not explore the underlying mechanisms behind the differences in mtDNAcn by race, other studies have reported multiple genome variants in mtDNA among black women compared with white women, which may account for these racial differences in mtDNAcn[27]. In addition, these mtDNA variants were likely a result of genetic adaptation to environmental factors and dietary changes experienced by black people due to historical migrations from Africa[27]. This genetic

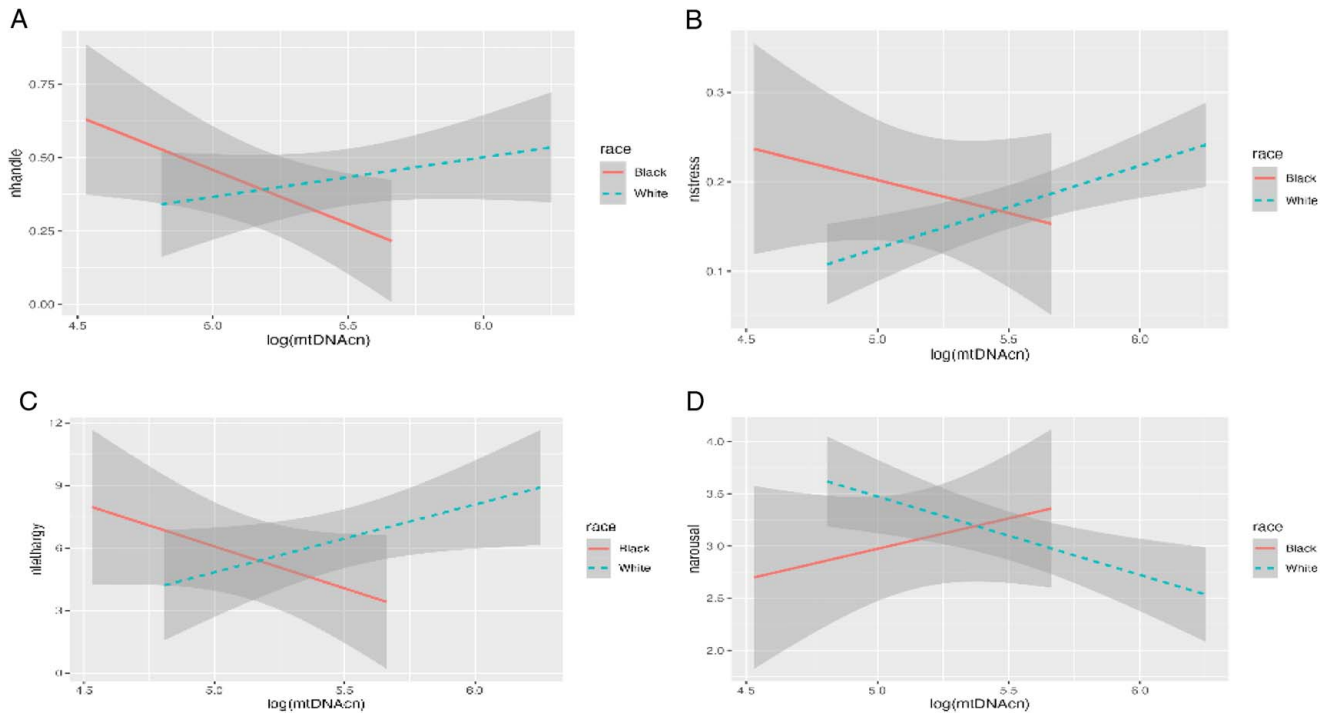

Figure 3. The association between mtDNAcn and neurobehavioral outcomes by race. In white infants, the positive associations between mtDNAcn and (A) nhandle ($P < 0.05$), (B) nstress ($P < 0.05$), (C) nlethargy ($P < 0.05$); and negative association between mtDNAcn and (D) arousal ($P < 0.01$) were observed. In black infants, the negative associations between mtDNAcn and (A) nhandle ($P < 0.05$), (B) nstress ($P < 0.05$), (C) nlethargy ($P < 0.05$); and positive association between mtDNAcn and (D) narousal ($P < 0.01$) were observed. mtDNAcn indicates mitochondrial DNA copy number.

adaptation could potentially increase the risk of diseases, such as ovarian cancer in black women[27,28].

In our study, the risk of PPROM has been linked to non-optimal performance such as high levels of stress, abstinence, excitability, and arousal in preterm infants. Although we did not identify a direct association between mtDNAcn and PPROM, existing evidence suggested that the maternal inheritance of elevated mtDNAcn may be a risk factor for PPROM[29,30]. Other studies have also suggested that maternal smoking or bacterial infection could lead to the overproduction of ROS and mitochondrial dysfunction, which may trigger an inflammatory response in the amniotic membrane and result in PPROM[31,32]. Furthermore, due to the placenta's limited filtering function, smoking-induced mitochondrial dysfunction is observed not only in smoking pregnant women but also in their infants[33], potentially impacting their health outcomes[34].

We did not observe significant sex-differentiated associations between mtDNAcn and NNNS or Bayley scores. However, we

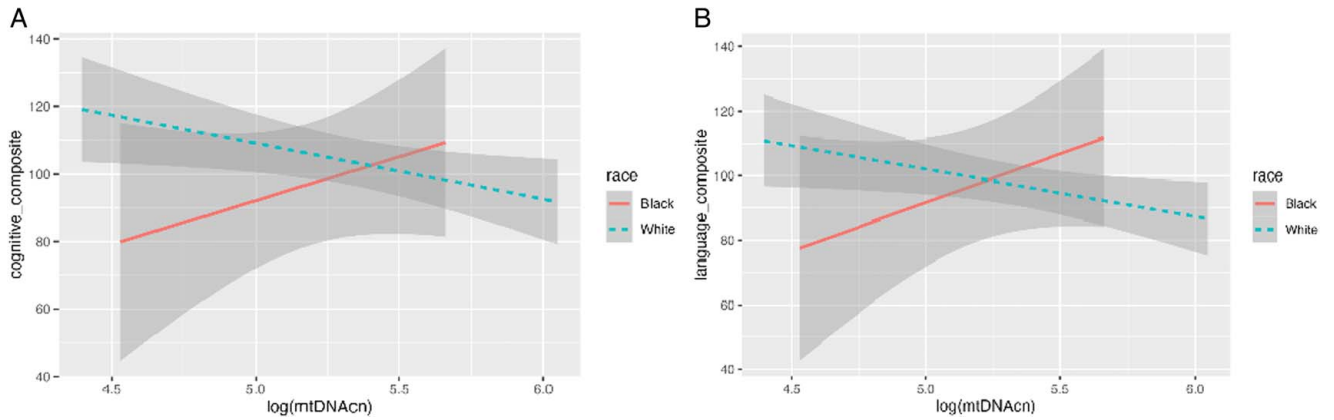

Figure 4. The association between mtDNAcn and neurodevelopmental outcomes in white and black infants. There was a negative association between mtDNAcn and cognitive composite scores in white infants and a positive association between mtDNAcn and cognitive composite scores in black infants (A). There was a significant negative association between mtDNAcn and language composite scores ($P < 0.05$) in white infants and a significant positive association between mtDNAcn and language composite scores ($P < 0.05$) in black infants (B). mtDNAcn indicates mitochondrial DNA copy number.

found that males had lower narousal scores at 36–38 PMA and lower cognitive and motor composite scores at 1 year of age compared with female cohorts, consistent with findings from other studies indicating that the male sex is an independent risk factor for poor neurodevelopmental outcomes[35]. The impaired neurodevelopment in male preterm infants may be attributed to factors such as their relatively lower energy and fat intake after birth[36], difficulties in adapting to maternal stress[37], the oxidative status of the placenta[38], and/or issues related to brain development[39].

Our study highlights the importance of assessing mtDNAcn in predicting neurobehavioral and neurodevelopmental outcomes in preterm infants during their early life. Our findings also suggest that mtDNAcn could serve as a clinical biomarker for identifying preterm infants at risk for neurobehavioral and neurodevelopmental disorders. Although the biomechanisms underlying the associations between mtDNAcn and neurobehavior or neurodevelopment are not yet fully understood, factors such as early life pain/stress, inflammation, maternal socioeconomic status, parental health, and other environmental influences[40,41] may affect the quantity of mtDNAcn. These potential confounders should be considered in future studies.

Conclusion

In summary, our study provides important supporting evidence for the established negative correlation between mtDNAcn and GA. In addition, it identified a race disparity in the correlation between mtDNAcn and neurobehavioral or neurodevelopmental outcomes in infants from birth until 2 years of CA. These findings suggest that elevated mtDNAcn holds potential as a biomarker for mitochondrial stress, perhaps associated with overproduction of ROS in preterm infants. However, due to the limited sample size ($n = 55$) in this longitudinal study, especially when comparing black and white infants, our findings may not be representative of the large population and may limit the generalizability of our results, as it might not capture the full range of variability and potential confounding factors present in a more diverse and extensive population. A follow-up investigation with a larger participant cohort is needed to strengthen the clinical significance of this data and to better understand underlying mechanisms. In addition, our study's reliance on a single measurement of mtDNAcn may overlook important signals, such as genes that regulate mtDNA levels. Measuring mtDNAcn across multiple time points would also be valuable, capturing the variations in mtDNAcn during early and later life in preterm infants. Future longitudinal approaches would help identify patterns and trends over time, offering a more comprehensive understanding of how early life experiences and racial disparities influence neurodevelopmental outcomes through changes in mtDNAcn.

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Conflict of interest disclosures

The authors declare that they have no financial conflict of interest with regard to the content of this report.

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