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Exploring Mitochondrial Heteroplasmy in Neonates: Implications for Growth Patterns and Overweight in the First Years of Life

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ABSTRACT

Background: Mitochondrial heteroplasmy reflects genetic diversity within individuals due to the presence of varying mitochondrial DNA (mtDNA) sequences, possibly affecting mitochondrial function and energy production in cells. Rapid growth during early childhood is a critical development with long-term implications for health and well-being. In this study, we investigated if cord blood mtDNA heteroplasmy is associated with rapid growth at six and 12 months and overweight in childhood at four to six years.

Methods: This study included 200 mother-child pairs of the ENVIR*ON*AGE birth cohort. Whole mitochondrial genome sequencing was performed to determine mtDNA heteroplasmy levels (in variant allele frequency; VAF) in cord blood. Rapid growth was defined for each child as the difference between WHO-SD scores of predicted weight at either six or 12 months and birth weight. Logistic regression models were used to determine the association of mitochondrial heteroplasmy with rapid growth and childhood overweight. Determinants of relevant cord blood mitochondrial heteroplasmies were identified using multiple linear regression models.

Results: One % increase in VAF of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy was associated with rapid growth at six months (OR=1.03; 95% CI: 1.01 to 1.05; p=0.001) and 12 months (OR=1.02; 95% CI: 1.00 to 1.03; p=0.02). Furthermore, this variant was associated with childhood overweight at four to six years (OR=1.01; 95% CI 1.00 to 1.02; p=0.05). Additionally, rapid growth at six months (OR=3.00; 95% CI: 1.49 to 6.14; p=0.002) and 12 months (OR=4.05; 95% CI: 2.06 to 8.49; p<0.001) was also associated with childhood overweight at four to six years. Furthermore, we identified maternal age, pre-pregnancy BMI, maternal education, parity, and gestational age as determinants of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy.

Conclusions: Our findings, based on mitochondrial DNA genotyping, offer insights into the molecular machinery leading to rapid growth in early life, potentially explaining a working mechanism of the development towards childhood overweight.

INTRODUCTION

Mitochondria, the powerhouses of our cells, are crucial for energy production through oxidative phosphorylation (OXPHOS). Each mitochondrion contains multiple copies of its own circular DNA, encoding various proteins essential for energy metabolism. Due to its proximity to the inner mitochondrial membrane where reactive oxygen species (ROS) are generated, mtDNA is more exposed to oxidative stress than nuclear DNA (1). The lack of protective histones and limited DNA repair mechanisms makes it more vulnerable to mutations (2). In fact, the mitochondrial mutation rate is about 100-fold higher than that of the nuclear genome (3). These mutations result in the presence of different mtDNA sequences within tissues or cells, called mitochondrial heteroplasmy (4). The association of mtDNA mutations and mitochondrial diseases has been studied before, but their role in more common, complex disorders has not been fully proven (5-8). Given the important role of mitochondria in energy metabolism and the fact that they are more prone to mutations, mitochondrial dysfunction contributes to cellular energy imbalance (9), possibly reduced energy expenditure, leading to various metabolic disorders, including obesity (10, 11). Cord blood mitochondrial heteroplasmy can potentially affect energy production (12). Previously, we identified an association between cord blood MT-ND4L$_{10550A>G}$ heteroplasmy and overweight in children aged four to six (13), but studies linking cord blood mitochondrial heteroplasmy and rapid growth are scarce. Changes in mitochondrial heteroplasmy might lead to enhanced mitochondrial function, resulting in increased ATP production. Another possibility is that mitochondrial heteroplasmy might lead to reduced energy expenditure, leading to metabolic disorders (10). Molecular pathways in cord blood have been found to mediate the risk of experiencing rapid growth at 12 months (14, 15), raising the question if cord blood mtDNA heteroplasmy could also affect rapid growth. As this concept is not well understood, we hypothesised that cord blood mtDNA heteroplasmy is associated with rapid growth at six and 12 months and childhood overweight at four to six years.

MATERIALS AND METHODS

STUDY POPULATION

Within the framework of the ongoing prospective birth cohort 'Environmental Influence on Aging in Early Life' (ENVIRONAGE; Flanders, Belgium) (16), we included 200 mother-child pairs recruited between February 2010 and September 2013 (17) (**Supplementary Figure S1**). Details on the study protocol have been described previously (16). Briefly, mothers without a planned caesarean section who can fill out a Dutch language questionnaire are eligible for participation. Biological samples (e.g., placental tissue, cord blood, maternal blood) are collected, and medical records during and after pregnancy can be accessed, which include foetal ultrasound data. In addition, lifestyle factors are derived from questionnaires filled out after delivery.

All study participants signed an informed consent according to procedures authorized by the Ethical Committees of the East-Limburg Hospital (Genk, Belgium) and Hasselt University. This study has been performed according to the Helsinki Declaration.

DATA COLLECTION

Medical records provided information about the date of birth, gestational age, newborn's sex, and birth weight. Pre-pregnancy BMI was determined between gestational weeks seven and nine. Questionnaires completed by the mothers provided detailed information on the participants. Maternal education was coded low (no diploma or primary school), middle (secondary school), or high (college or university). Parity was divided into three categories: primiparous (first child), secundiparous (second child), or multiparous (third child or more). Smoking during pregnancy was coded "yes", in case the mother reported that she had smoked during the pregnancy, otherwise as "no". Newborn's ethnicity was considered European when at least two grandparents were European, otherwise as non-European. In addition, trained staff of the governmental agency "Kind en Gezin" (K&G – Child and Family, URL:

https://www.kindengezin.be/en/child-and-family) reported child weight at an average of seven time points in the first year. Predicted weight at six or 12 months was calculated using mixed-effects linear regression models with the best fitting fractional polynomials of age by sex (18). WHO's growth curves were used to calculate the sex- and age-adjusted standard deviation (SD) weight scores. Rapid growth (yes/no) was defined as the difference between WHO-SD scores of predicted weight at six or 12 months and birth weight being greater than 0.67, according to Ong et al. (19). At the follow-up visit between four and six years of age, trained examiners measured clinical parameters of the child, including height and weight (of which BMI was calculated as the ratio between weight and squared height), and mothers filled out multiple questionnaires addressing general information about the lifestyle of the child and parents. Childhood overweight was defined as WHO-SD BMI scores being higher than the sex- and age-specific BMI cut-offs, according to the International Obesity Task Force (IOTF) (20).

NEXT GENERATION SEQUENCING

Umbilical cord blood was collected in Vacutainer® Plus Plastic K2EDTA Tubes (BD, Franklin Lakes, NJ, USA) right after delivery. Buffy coats containing cord blood leukocytes were used for the extraction of total genomic DNA with the QIAamp DNA Mini Kit (Qiagen, Venlo, the Netherlands) following the manufacturer's instructions. DNA quantity and purity were determined on a NanoDrop ND-1000 UV-Vis spectrometer (Thermo Scientific, Wilmington, DE). Total genomic DNA samples was stored at -80°C for further analyses.

Whole mitochondrial genome sequencing was performed by Macrogen Europe, as described elsewhere (17). Demultiplexed fastq files were used for downstream analyses, which were performed with Geneious Prime (version 2021.2.2). Adapters and low-quality regions were trimmed using BBDuk, after which sequences were aligned to the human mitochondrial reference genome (NC_012920, URL: https://www.ncbi.nlm.nih.gov/nuccore/NC_012920), and variants were called. Base call accuracy was

assessed with the Phred Score. Mitochondrial heteroplasmy levels were determined based on the variant allele frequency (VAF). This study focused on SNPs with a VAF >5%, coverage >300x, and a prevalence of at least 5% in the study population. SNPs without variation were excluded.

MITOCHONDRIAL DNA CONTENT

Cord blood mtDNA content was measured by determining the ratio of two mitochondrial gene copy numbers (MTF3212/R3319 and *MT-ND1*) to two single-copy nuclear control genes (*RPLP0* and *ACTB*) using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The full protocol is described elsewhere (21).

STATISTICAL ANALYSES

The power calculations were performed using G*Power (version 3.1.9.7) and based on the observed association between cord blood MT-ND4L$_{10550A>G}$ heteroplasmy and childhood overweight (13). Considering $\beta = 95\%$ and $\alpha = 0.05$, a sample size of n = 200 was sufficient. Data management and statistical analysis were performed using R (version 4.3.1) and RStudio software (version 2023.06.0.0). Continuous variables (i.e., maternal age, pre-pregnancy BMI, gestational age, birth weight, cord blood mtDNA content, and children's age at follow-up) were presented as means ± SD, and categorical variables (i.e., maternal education, parity, smoking during pregnancy, newborn's sex, ethnicity, rapid growth, and childhood overweight) as numbers (frequency in %).

Firstly, the association between cord blood mitochondrial heteroplasmy and rapid growth was evaluated using logistic regression models (i) minimally adjusted for gestational age and newborn's sex and (ii) fully adjusted for covariates selected based on previous literature (13, 22, 23), including maternal age, maternal education, pre-pregnancy BMI, parity, smoking during pregnancy, gestational age, newborn's sex, ethnicity, birth weight, and cord blood mtDNA content. Cord blood mtDNA content was added to correct for possible variation in mtDNA input. Estimates were provided as odds ratios (OR) with 95% CI of developing rapid growth per 1% increase in VAF. Furthermore, the association between cord blood mtDNA

content and rapid growth was explored using logistic regression models adjusted for maternal age, maternal education, pre-pregnancy BMI, parity, smoking during pregnancy, gestational age, newborn's sex, ethnicity, birth weight, cord blood leukocyte count, and cord blood platelet count. Estimates were provided as OR with 95% CI of developing rapid growth per unit increase in cord blood mtDNA content. The residuals of the regression models, as well as a visual inspection of histograms and QQ-plots were used to evaluate the normality assumption and they did not deviate from normality. Principal component analysis was performed to determine the degree of correlation between the detected SNPs (17, 24). To allow for making multiple comparisons while considering the degree of correlation, the effective number of tests based on principal component analysis was estimated. Since 11 principal components explained 90% of the variation in the data (**Supplementary Table S1**), a corrected significance level of $p = 0.05/11 = 0.005$ was used. In sensitivity analyses, we examined the association between cord blood mitochondrial heteroplasmy and rapid growth after additionally adjusting for leukocyte and platelet count and stratified by sex.

Secondly, based on the observed significant results, the association between cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy and childhood overweight was determined with logistic regression models adjusted for the same set of covariates and, in addition, the child's age at follow-up. Estimates were provided as OR with 95% CI of childhood overweight per 1% increase in VAF. Furthermore, the association between rapid growth and childhood overweight was assessed. Estimates were provided as OR with 95% CI of having childhood overweight after experiencing rapid growth at six or 12 months. For both analyses, a p-value < 0.05 was considered significant.

Lastly, possible determinants of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy were investigated using multiple linear regression models adjusted for maternal age, maternal education, pre-pregnancy BMI, parity, smoking during pregnancy, newborn's sex, ethnicity, gestational age, birth weight, and cord blood mtDNA content. Estimates for maternal age, pre-pregnancy BMI, gestational age, birth weight, and cord

149 blood mtDNA content were provided as difference in VAF (in %) with 95% CI per unit increase of the
150 characteristic. Estimates for maternal education, smoking during pregnancy, parity, sex, and ethnicity were
151 provided as difference in VAF (in %) with 95% CI compared to low education, no smoking during pregnancy,
152 primiparity, male, and European, respectively. As this analysis was exploratory, a significance level of $p <$
153 0.10 was used.

154 # RESULTS

155 ## POPULATION CHARACTERISTICS

156 An overview of the study population characteristics is provided in **Table 1**. Participating mothers were 29.7
157 ± 4.1 years old, had a pre-pregnancy BMI of 24.1 ± 4.5 kg/m², and most of them were highly educated
158 (66.5%). Most pregnancies were primiparous (53.5%), and gestation lasted 39.6 ± 1.4 weeks. Newborns
159 weighed 3420 ± 424g at birth, 52.0% were girls, and most were of European ethnicity (94.0%). The
160 assessment of children's growth revealed that 12.0% and 27.5% of them showed rapid growth at six and
161 12 months, respectively. At the follow-up visit, the children were 4.7 ± 0.4 years old and 15.5% of them
162 were overweight.

163 **Table 1: Study population characteristics (n = 200).**

Characteristic	Mean ± SD or n (%)
Maternal	
Age at delivery (years)	29.7 ± 4.1
Pre-pregnancy BMI (kg/m²)	24.1 ± 4.5
Maternal education	
Low	12 (6.0)

Middle	55 (27.5)
High	133 (66.5)
Parity	
Primiparous	107 (53.5)
Secundiparous	69 (34.5)
Multiparous	24 (12.0)
Smoking during pregnancy (yes)	24 (12.0)
Newborn	
Gestational age (weeks)	39.6 ± 1.4
Sex (female)	104 (52.0)
Birth weight (g)	3420 ± 424
Ethnicity	
European	188 (94.0)
Non-European	12 (6.0)
Child at follow-up	
Age (years)	4.7 ± 0.4
Childhood overweight (yes)	31 (15.5)
Rapid growth (yes)	
At 6 months	24 (12.0)
At 12 months	55 (27.5)

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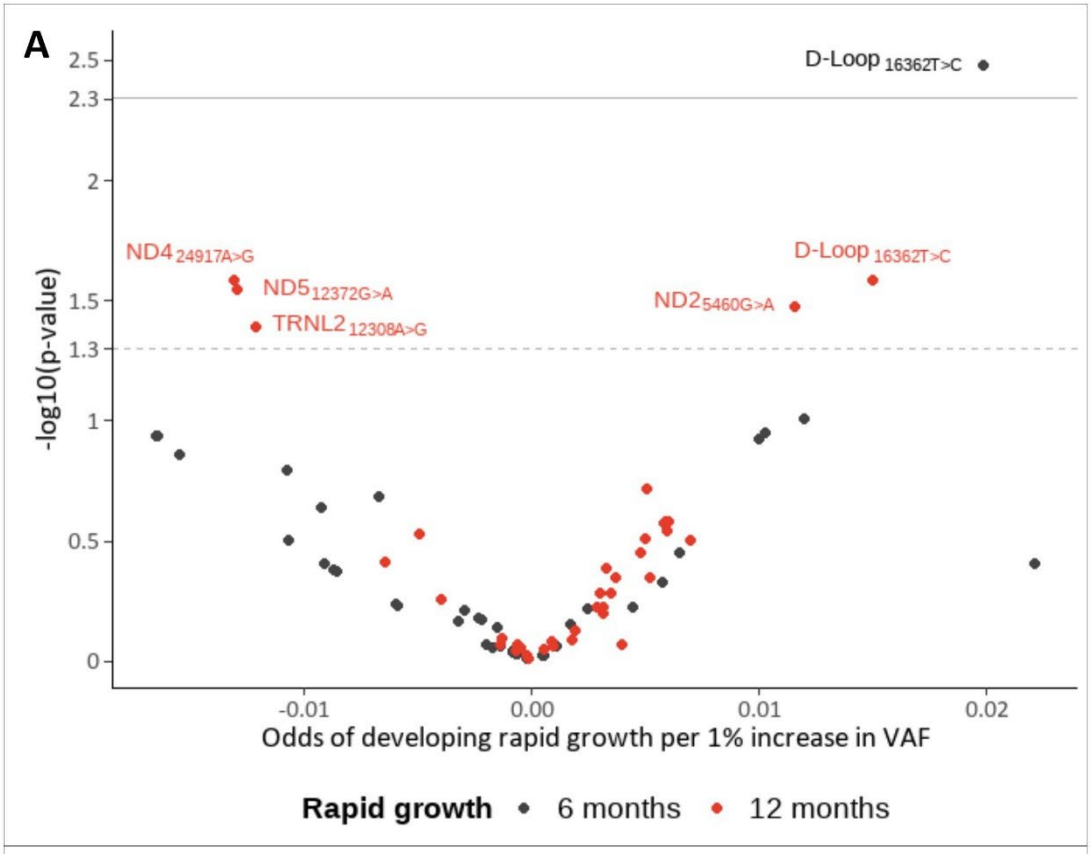
THE ASSOCIATION BETWEEN CORD BLOOD MITOCHONDRIAL HETEROPLASMY, mtDNA CONTENT, AND RAPID GROWTH

In our study population, 2928 cord blood mtDNA variants were detected, including 2866 SNPs (**Supplementary Figure S2**). Considering the exclusion criteria (n = 2818) and the exclusion of SNPs due to lack of variation (n = 6), 42 mtDNA SNPs were included for analyses (**Supplementary Table S2**). In the minimally adjusted model corrected for gestational age and newborn's sex, cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy was significantly associated with rapid growth at six months (OR = 1.02; 95% CI: 1.01 to 1.03; p = 0.003) after correction for multiple testing (**Figure 1A**). Cord blood MT-ND4$_{24917A>G}$, MT-ND5$_{12372G>A}$, MT-TRNL2$_{12308A>G}$, MT-ND2$_{5460G>A}$, and MT-D-Loop$_{16362T>C}$ heteroplasmy tended to be linked with rapid growth at 12 months ($0.005 < p < 0.05$) (**Figure 1A**). After full adjustment and a correction for multiple testing, cord blood MT-D-Loop$_{16362T>C}$, on average 5.5% ± 22.8% (**Supplementary Table S2**), was significantly associated with rapid growth at six months (OR = 1.03; 95% CI: 1.01 to 1.05; p = 0.001) and 12 months (OR = 1.02; 95% CI: 1.00 to 1.03; p = 0.02). The latter did not survive significance after correction for multiple testing. The same trend was found for the association between cord blood MT-ND2$_{5460G>A}$, on average 5.2% ± 21.9% (**Supplementary Table S2**), and rapid growth at six months (OR = 1.02; 95% CI: 1.00 to 1.04; p = 0.04) (**Figure 1B**). Sensitivity analyses for the association between cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy and rapid growth at six months showed that additionally adjusting for leukocyte and platelet count did not affect our results (**Supplementary Table S3**). Furthermore, stratifying by sex did not affect the effect size, but the association was stronger in girls (p = 0.006) compared to boys (p = 0.05; **Supplementary Table S3**). Cord blood mtDNA content was not linked with rapid growth at six or 12 months (**Supplementary Table S4**).


A
D-Loop 16362T>C
ND4 24917A>G
ND5 12372G>A
TRNL2 12308A>G
ND2 5460G>A
D-Loop 16362T>C
-log10(p-value)
Odds of developing rapid growth per 1% increase in VAF
Rapid growth
6 months
12 months

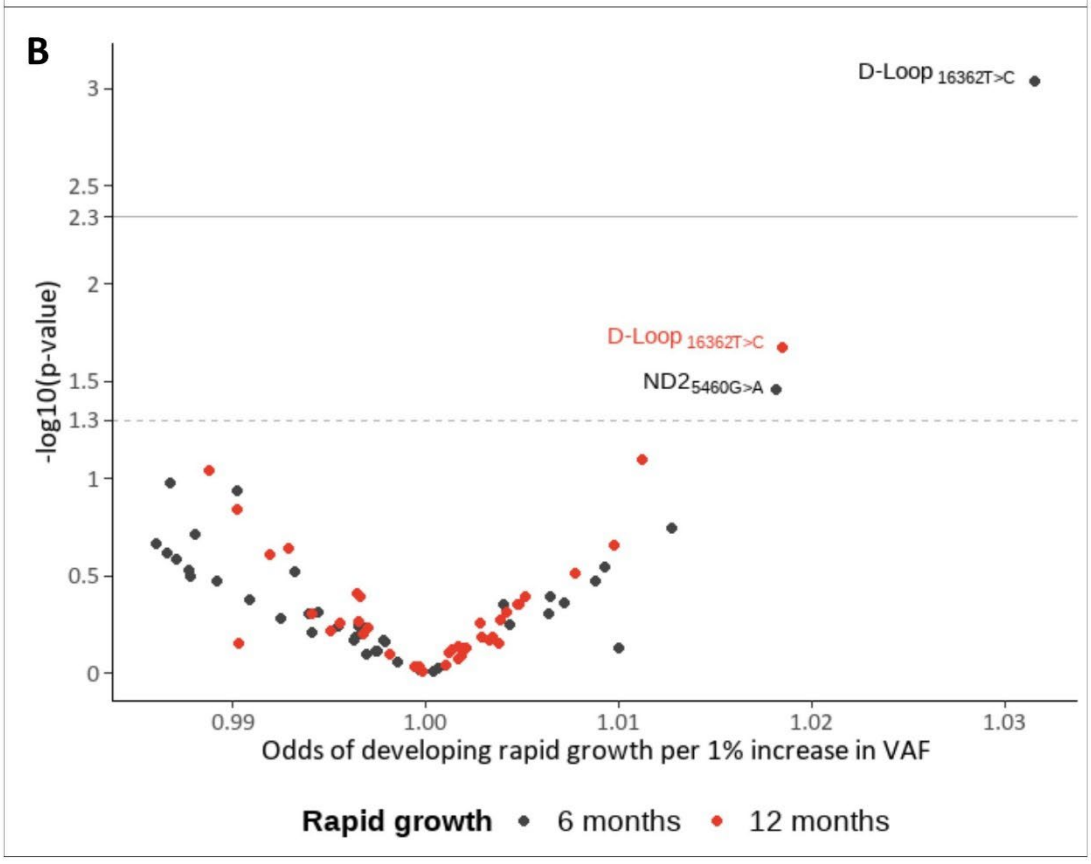

B
D-Loop 16362T>C
D-Loop 16362T>C
ND2 5460G>A
-log10(p-value)
Odds of developing rapid growth per 1% increase in VAF
Rapid growth
6 months
12 months

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Figure 1: Volcano plot for the association between cord blood mitochondrial heteroplasmy and rapid growth at six (black) and 12 months (red). (A) In the minimally adjusted model corrected for gestational age and newborn's sex, MT-D-Loop$_{16362T>C}$ heteroplasmy was significantly associated with rapid growth at six months ($p < 0.005$; grey solid line). MT-ND4$_{24917A>G}$, MT-ND5$_{12372G>A}$, MT-TRNL2$_{12308A>G}$, MT-ND2$_{5460G>A}$, and MT-D-Loop$_{16362T>C}$ heteroplasmy tended to be linked with rapid growth at 12 months ($p < 0.05$; grey dotted line). Estimates were provided as OR of developing rapid growth per % increase in VAF. **(B)** In the fully adjusted model corrected for maternal age, maternal education, pre-pregnancy BMI, parity, smoking during pregnancy, gestational age, newborn's sex, ethnicity, birth weight, and cord blood mtDNA content, MT-D-Loop$_{16362T>C}$ heteroplasmy was significantly associated with rapid growth at six months ($p < 0.005$; grey solid line), with a similar trend at 12 months ($p < 0.05$; grey dotted line). MT-ND2$_{5460G>A}$ heteroplasmy tended to be linked with rapid growth at six months ($p < 0.05$; grey dotted line). Estimates were provided as OR of developing rapid growth per % increase in VAF.

THE ASSOCIATION BETWEEN CORD BLOOD MT-D-LOOP$_{16362T>C}$ HETEROPLASMY, RAPID GROWTH, AND CHILDHOOD OVERWEIGHT

Cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy was positively associated with childhood overweight at four to six years, although borderline significant. One % increase in VAF for cord blood MT-D-Loop$_{16362T>C}$ was linked with higher odds of a four-to-six-year-old child being overweight (OR = 1.01; 95% CI 1.00 to 1.02; $p = 0.05$) after adjustment for maternal age, maternal education, pre-pregnancy BMI, parity, smoking during pregnancy, gestational age, newborn's sex, ethnicity, birth weight, cord blood mtDNA content, and the child's age at follow-up. Experiencing rapid growth at six months of age increased the odds of having childhood overweight at four to six years (OR = 3.00; 95% CI: 1.49 to 6.14; $p = 0.002$). Furthermore, rapid growth at 12 months of age was associated with increased odds of developing childhood overweight at four to six years (OR = 4.05; 95% CI: 2.06 to 8.49; $p < 0.001$).

DETERMINANTS OF CORD BLOOD MT-D-LOOP$_{16362T>C}$ HETEROPLASMY

Five characteristics, specifically maternal age, pre-pregnancy BMI, maternal education, parity, and gestational age were identified as determinants of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy (**Table 2**), with a significance level set at $p < 0.10$. One year increase in maternal age was associated with an increase of 0.85% (95% CI: -0.02 to 1.72; $p = 0.05$) of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy. One unit increase in pre-pregnancy BMI was linked with an increase of 0.63% (95% CI: -0.08 to 1.34; $p = 0.08$) of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy. Middle and high maternal education levels were associated with a -17.26% (95% CI: -31.95 to -2.57; $p = 0.02$) and -17.15% (95% CI: -31.55 to -1.75; $p = 0.02$) lower cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy compared to low maternal education, respectively. Multiparity was associated with a decrease of 12.86% (95% CI: -24.12 to -1.61; $p = 0.03$) when compared to primiparity, but no difference was found between secundi- and primiparity. One day increase in gestational age was linked with a decrease of 0.32% (95% CI: -0.69 to 0.06; $p = 0.10$) of cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy.

Table 2: Determinants for cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy. Models included maternal age, pre-pregnancy BMI, maternal education, smoking during pregnancy, parity, newborn's sex, ethnicity, gestational age, birth weight, and cord blood mtDNA content. Estimates for maternal age, pre-pregnancy BMI, gestational age, birth weight, and cord blood mtDNA content were provided as difference in VAF (in %) with 95% CI per unit increase of the characteristic. Estimates for maternal education, smoking during pregnancy, parity, sex, and ethnicity were provided as difference in VAF (in %) with 95% CI compared to low education, no smoking during pregnancy, primiparity, male, and European, respectively. $p < 0.10$ was considered significant.

	n	Estimate (95% CI)	*p*-value
Maternal age (years)	200	0.85 (-0.02 to 1.72)	0.05
Pre-pregnancy BMI (kg/m²)	200	0.63 (-0.08 to 1.34)	0.08

Maternal education			
Low	12	Reference	
Middle	55	-17.26 (-31.95 to -2.57)	0.02
High	133	-17.15 (-31.55 to -1.75)	0.02
Smoking during pregnancy			
No	176	Reference	
Yes	24	-3.15 (-13.63 to 7.33)	0.55
Parity			
Primiparous	107	Reference	
Secundiparous	69	-2.45 (-9.88 to 4.97)	0.52
Multiparous	24	-12.86 (-24.12 to -1.61)	0.03
Sex			
Male	96	Reference	
Female	104	0.14 (-6.35 to 6.64)	0.97
Ethnicity			
European	188	Reference	
Non-European	12	3.14 (-10.51 to 16.78)	0.65
Gestational age (days)	200	-0.32 (-0.69 to 0.06)	0.10
Birth weight (g)	200	0.002 (-0.01 to 0.01)	0.74
Cord blood mtDNA content	200	-1.56 (-6.90 to 3.79)	0.57

DISCUSSION

The key findings of our study include a link between cord blood mtDNA heteroplasmy and growth patterns during the first year of life. Specifically, we observed a significant positive association between cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy and accelerated growth at six months, with a consistent trend persisting at the twelve-month mark. Further, a higher level of this genetic variant was correlated with childhood overweight between the ages of four and six, and pre-pregnancy BMI, parity, and maternal education were recognized as potential determinants.

Infants' rapid growth, or catch-up growth, was first described as accelerated growth in response to recovery from illness or starvation (25, 26). However, a study involving twins revealed that genetic factors also played a role in determining weight at six months and the rate of weight gain during infancy (27). Furthermore, infants genetically predisposed towards higher weight gain but born to lean mothers showed rapid postnatal growth (28). To unravel the underlying mechanisms of rapid growth, both environmental and genetic factors should be considered. One potential underlying mechanism might be mitochondrial heteroplasmy, which refers to the presence of multiple mtDNA sequences within an individual's cells or tissues (4). The thrifty phenotype hypothesis states that adverse prenatal exposures have the potential to lead to changes in fetal metabolism (29), postulating that alterations in mitochondrial biology might occur. These changes can contribute to the storage of excess calories, predisposing children to develop overweight or obesity (29). For instance, prenatal air pollution exposure was linked with changes in cord blood mtDNA heteroplasmy levels (13, 17), of which cord blood MT-ND4L$_{10550A>G}$ heteroplasmy was found to be positively associated with childhood overweight at four to six years (13).

We identified a link between rapid growth at six months and cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy, which is a variant in the hypervariable, non-coding displacement loop (i.e., D-Loop). The D-Loop is an important region in the mtDNA, as it regulates replication and transcription of mitochondrial genes (30). It consists of three hypervariable (HV) regions (31), where the MT-D-Loop$_{16362T>C}$ variant is located in HV

region I (HVI). Consistent with our results, mitochondrial heteroplasmy in this region was linked with metabolic disorders in early life (32) and adulthood (32, 33). In the ALSPAC cohort, another variant (MT-D-Loop$_{16189T>C}$) was linked with a lower ponderal index at birth, while after two years, no difference in adiposity was found between children with the variant compared to those without it. The authors suggested that children with this MT-D-Loop$_{16189T>C}$ variant showed early postnatal rapid growth (32), however, the association between MT-D-Loop$_{16189T>C}$ heteroplasmy and rapid growth was not confirmed in our study (data not shown). This specific mitochondrial variant was also associated with insulin resistance (33) and a 5-fold increase in type 2 diabetes in the Hertfordshire cohort (32). Our study demonstrated that rapid growth was influenced by cord blood mitochondrial heteroplasmy rather than cord blood mtDNA content. This finding suggests that not the amount of mitochondrial DNA but mitochondrial mutations might play a more important role in rapid growth development. As also cord blood metabolites have been linked with rapid growth in the first 12 months of life and childhood overweight (14, 18), our research adds to this knowledge by identifying cord blood markers that have the potential to predict the risk of developing rapid growth in the early phases of life.

We showed a borderline significant association between cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy and childhood overweight at four to six years (p = 0.05). The study by Flaquer et al. (34) showed a link between mitochondrial variants in multiple genes (i.e., *MT-COX1, MT-COX3, MT-ND1, MT-ND2,* and *MT-ND4L*) and BMI in adults (34), while another study did not find an association with common mtDNA variants (9). Two variants in the D-Loop region (MT-D-Loop$_{16292C>T}$ and MT-D-Loop$_{16189T>C}$) were linked with obesity in adults (10). Consistent with our results, we previously found that cord blood MT-ND4L$_{10550A>G}$ heteroplasmy was positively linked with childhood overweight at four to six years (13). In contrast, another study did not find an association between mitochondrial heteroplasmy and childhood obesity (6). As the D-Loop is an important regulator of the mtDNA (30), it is plausible that it plays a role in the development of overweight

or obesity. However, the exact mechanism underlying the link between mitochondrial heteroplasmy and childhood overweight warrants further investigation.

Several studies reported the link between rapid growth and metabolic disorders, such as obesity (19), cardiovascular diseases (35-38), and type 2 diabetes (35, 37) in later life. Especially growth trajectories in the first year of life may be critical for long-term metabolic health. Rapid growth between three and 12 months of life was linked with higher insulin resistance (39). Between zero and six months of life, rapid growth predicted metabolic risk in adolescents aged 17 years old (40). Our study demonstrated a link between rapid growth at six and 12 months of life and developing overweight at four to six years of age. Consistent with our results, experiencing rapid growth at 12 months of age was associated with higher odds of being overweight at 16 and 18 years old (41), and rapid growth in the first five years of life was linked with a higher BMI in young adulthood (42). Furthermore, a meta-analysis showed that rapid growth in infancy was associated with overweight and obesity from childhood to adulthood (43). They also reported that rapid growth from birth to 12 months was linked with a higher likelihood of childhood overweight and obesity compared to rapid growth from birth to two years (43). Taken together, these data suggest that the first few years of life are a critical time window for monitoring growth patterns in infants.

To investigate factors influencing cord blood mitochondrial heteroplasmy, our study examined several possible determinants of which four maternal variables (i.e., maternal age, pre-pregnancy BMI, maternal education, and parity) were linked with cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy. A previous study found that higher pre-pregnancy BMI, low maternal education, and primiparity were also positively linked with childhood overweight at four to six years old (14). Parity was also found to be a potential determinant for infant rapid growth, while no association was found with pre-pregnancy BMI and maternal education (22). Since mtDNA is exclusively inherited from the mother (3), any mtDNA mutations can potentially be transmitted to her offspring. A positive association was found between maternal age at fertilisation and mitochondrial heteroplasmy in their child (44). Furthermore, several maternal factors have been known

to affect mitochondrial function in their offspring. Pre-pregnancy BMI altered the expression of genes involved in mitochondrial metabolism in umbilical vein endothelial cells (45). Moreover, maternal allostatic load (including pre-pregnancy BMI) was linked with mtDNA content and mitochondrial bioenergetic capacity in early childhood (46). Oxidative stress during pregnancy is influenced by lifestyle factors, such as socioeconomic status (47). In addition, animal studies showed that parity influenced mitochondrial enzyme activity (48) and respiratory capacity (49). Furthermore, we found that gestational age was linked with cord blood MT-D-Loop$_{16362T>C}$ heteroplasmy, which can be explained due to an increase in oxidative stress as gestation progresses (50). These findings suggest an important role for these factors in mitochondrial function.

In the context of the Developmental Origins of Health and Disease (DOHaD) hypothesis, having distinct mitochondrial heteroplasmies at birth could contribute to potential health effects in later life, for example, metabolic diseases. The translational potential of our results lies in identifying cord blood mitochondrial heteroplasmy as a potential predictor of the risk of developing rapid growth in the early phases of life, which in turn may lead to a higher risk for childhood obesity. This marker, in combination with known risk factors, may offer the possibility of contributing to a valuable screening tool for early interventions. Prior to translating this knowledge into a broader context, it is essential to explore the causality of our identified associations through longitudinal and experimental studies.

Strengths and limitations – Our study has several strengths. We used whole mitochondrial genome sequencing to analyse the entire mitochondrial genome. With an average coverage of 19,627x, base calls were made with a high degree of confidence. We investigated the link between multiple mtDNA variants, which are potentially correlated. Therefore, we used principal component analysis to correct the significance level for the analyses between mitochondrial heteroplasmy and rapid growth. We acknowledge some limitations in our study. Firstly, our study has a relatively small sample size (n = 200), which prevents us from stratifying our analyses, for example, by breastfeeding practices. Secondly, in this

study, we focused on maternal characteristics, while paternal factors might also contribute to the growth patterns of the child (51, 52), although not via mitochondrial heteroplasmy. While we showed a link between cord blood mitochondrial heteroplasmy and growth patterns in early life, the effect size is relatively small, underscoring the need for additional research to explore this association further.

CONCLUSION

We presented evidence that mitochondrial heteroplasmy in neonates is associated with rapid growth in infancy and overweight in early childhood, providing new insights into a potential mechanism underlying growth patterns in the first years of life. In addition, multiple factors (i.e., maternal age, pre-pregnancy BMI, maternal education, parity, and gestational age) were found to influence mitochondrial heteroplasmy in neonates. Understanding the relationship between mitochondrial heteroplasmy, energy imbalance, and growth patterns requires further investigation into specific molecular mechanisms that link mitochondrial function to growth regulation. Additionally, longitudinal studies assessing growth trajectories in individuals with varying levels of heteroplasmy could provide valuable insights into the link between mitochondrial dynamics, rapid growth, and childhood overweight.

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AUTHOR CONTRIBUTIONS

Charlotte Cosemans: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **Rossella Alfano**: Data Curation, Writing – Review & Editing. **Hanne Sleurs**: Investigation, Writing – Review & Editing. **Dries S Martens**: Investigation, Writing – Review & Editing. **Tim S Nawrot**: Conceptualization, Funding acquisition, Writing – Review & Editing, Supervision. **Michelle Plusquin**: Conceptualization, Writing – Review & Editing, Supervision.

ROLE OF THE FUNDING SOURCE

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the author(s) used ChatGPT in order to improve the language and readability of the manuscript. After using this tool, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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