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In utero exposure to air pollutants and mitochondrial heteroplasmy in neonates

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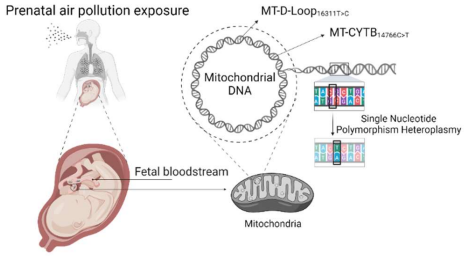
ABSTRACT ART



ABSTRACT

Mitochondria are sensitive to oxidative stress, which can be caused by traffic-related air pollution. Placental mtDNA mutations have been previously linked with air pollution. However, the relationship between prenatal air pollution and cord blood mtDNA mutations has been poorly understood. Therefore, we hypothesized that prenatal $PM_{2.5}$ and NO_2 exposures are associated with cord blood mtDNA heteroplasmy. As part of the ENVIRONAGE cohort, 200 mother-newborn pairs were recruited. Cord blood mitochondrial SNPs were identified by whole mitochondrial genome sequencing and heteroplasmy levels

were evaluated based on the variant allele frequency (VAF). Outdoor $PM_{2.5}$ and NO_2 concentrations were determined by a high-resolution spatial-temporal interpolation method, based on the maternal residential address. Distributed lag linear models (DLMs) were used to determine sensitive time windows for the association between NO_2 exposure and cord blood mtDNA heteroplasmy. A 5 μg/m³ increment in NO_2 was linked with MT-D-Loop$_{16311T>C}$ heteroplasmy from gestational week 17–25. MT-CYTB$_{14766C>T}$ was negatively associated with NO_2 exposure in mid pregnancy, from week 14–17, and positively associated in late pregnancy, from week 31–36. No significant associations were observed with prenatal $PM_{2.5}$ exposure. This is the first study to show that prenatal NO_2 exposure is associated with cord blood mitochondrial mutations and suggests two critical windows of exposure in mid- to late-pregnancy.

SYNOPSIS

Prenatal NO_2 exposure is associated with cord blood mitochondrial D-Loop$_{16311T>C}$ and CYTB$_{14766C>T}$ heteroplasmy in mid- to late-pregnancy.

KEYWORDS: mitochondria, SNP, heteroplasmy, air pollution, DLM

INTRODUCTION

Mitochondria, double-membraned cellular organelles, are responsible for ATP production and serve as central regulators of metabolism and oxidative stress. Each mitochondrion carries 2 – 10 copies of mitochondrial DNA (mtDNA) (1). Mitochondria are susceptible to mutations, since they have limited repair mechanisms and lack the protection of histones (2). Multiple mtDNA mutations have been described to date. These mutations are known to be involved in several mitochondrial diseases, like mitochondrial diabetes mellitus (MDM) (3, 4), maternally inherited diabetes deafness (MIDD) (5, 6), mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (3, 4), and Leigh's disease (3, 7). Mutations in the mtDNA sequence can lead to a heterogenic population of sequences, called heteroplasmy, which is defined as the mixed population of mtDNA sequences present in tissues or cells (5,

8, 9). Several studies showed that higher levels of heteroplasmy were related to several diseases (8-10). A minimum number of mutated mtDNA sequences is necessary before mitochondrial dysfunction will appear, which is the so-called threshold effect. This threshold ranged between 60-90% mutant to wild type mtDNA, dependent on the mutation and tissue (11). Heteroplasmy levels can accumulate, leading to impaired mitochondrial function, affecting human health (12). One study reported that the mutational load in mtDNA of placental tissue, in particular in genes coding NADH dehydrogenase and subunits of ATP synthase, was associated with prenatal exposure to fine particulate matter ($PM_{2.5}$) (13).

Mitochondria are sensitive to damage by oxidative stress, which can be caused by traffic-related air pollution such as $PM_{2.5}$ and nitrogen dioxide (NO_2) (14). $PM_{2.5}$ exposure was significantly associated with mitochondrial 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels, a biomarker for oxidative stress, in maternal blood during the entire pregnancy (15). In addition, a 10 µg/m³ increment in NO_2 exposure during pregnancy was associated with a 4.9% decrease in placental mtDNA content (16). Prenatal NO_2 exposure also inversely affected infant growth parameters, such as height at six months of age, which could be mediated by placental mtDNA content (17). Furthermore, NO_2 exposure in rats impaired the mitochondrial energy metabolism, including reduced ATP production and increased reactive oxygen species (ROS) production, as well as inhibition of mitochondrial biogenesis (18). Since there has been a considerable increase of evidence reporting the influence of air pollution on human health, the World Health Organization (WHO) updated their air quality guidelines (AQG) in September 2021 (19). The European Environment Agency (EEA) monitors $PM_{2.5}$ levels in 340 European cities (20). Based on the measurements of 2020 and 2021, the $PM_{2.5}$ concentrations in Flanders, Belgium ranged from 10.4 to 12.2 µg/m³, which is classified as moderate air quality (WHO AQG). The Belgian annual mean NO_2 level in 2019 respected the threshold of the EU (i.e., Air Quality Directive 2008/50/EC), but was almost double of the NO_2 WHO guidelines (21).

Although the association between air pollution and mitochondria has been studied before, the role of prenatal air pollution exposure on cord blood mitochondrial mutations is poorly understood. As cord blood reflects the state of the foetus and its mitochondrial makeup at the start of life (13), having insight into this mechanism can unravel complex mechanisms of disease onset related to mitochondrial function, such as neurodegenerative disorders (22, 23), and thereby contribute to the "Developmental Origins of Health and Disease" hypothesis (DOHaD) that states that health or diseases may find their origin in early life (24). Therefore, we hypothesized that *in utero* exposure to $PM_{2.5}$ and NO_2 is linked with mitochondrial heteroplasmy in cord blood of newborns.

MATERIALS AND METHODS

STUDY POPULATION

This study is part of the ongoing prospective Environmental Influence on Aging in Early Life (ENVIR*ON*AGE) birth cohort (Flanders, Belgium) and recruited 200 mother-newborn pairs between February 2010 and September 2013 (**Supplementary Figure S1**). Details on the enrolment of eligible mother-newborn pairs are available in supplemental information.

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

DATA AND SAMPLE COLLECTION

Immediately after delivery, umbilical cord blood was sampled in Vacutainer® Plus Plastic K2EDTA Tubes (BD, Franklin Lakes, NJ, USA) and centrifuged for 15 min at 3200 rpm to retrieve buffy coats containing cord blood leukocytes for DNA extraction. Samples were kept at -80°C for future analyses.

90 The obstetrician recorded data at birth, including newborn's sex and gestational age. The medical records
91 provided information on maternal age, pre-pregnancy BMI, and date at delivery. Between gestational
92 weeks 7 – 9, maternal pre-pregnancy BMI (kg/m^2) was registered. In addition, a questionnaire completed
93 by the mothers addressed their health and lifestyle status before and during pregnancy. They provided
94 information about their smoking status, maternal education, parity, and the newborn's ethnicity, as well
95 as their residential address. Maternal smoking during pregnancy was coded "yes", otherwise "no".
96 Socioeconomic status was based on maternal education and labelled low (no diploma or primary school),
97 middle (secondary school), or high (college or university). If at least two of the neonate's grandparents
98 were European, the newborn's ethnicity was labelled as "European", otherwise "non-European". Parity
99 was coded primi-, secundi-, or multiparous. Based on the date at delivery, season of delivery was divided
100 in two categories, namely "warm" (April 1st – September 30th) or "cold" (October 1st – March 31st).

101 PRENATAL AIR POLLUTION EXPOSURE ASSESSMENT

102 To determine the outdoor $PM_{2.5}$ and NO_2 concentrations (in $\mu g/m^3$), a high-resolution spatial-temporal
103 interpolation method based on the maternal residential address during pregnancy was used (25),
104 considering address changes of mothers during the pregnancy period. Details on the assessment of air
105 pollution exposure are available in supplemental information. To evaluate possible critical exposure
106 windows, residential air pollution levels were determined for particular time periods during pregnancy,
107 specifically for the weekly average, for each trimester of pregnancy (i.e., date of conception – 13
108 gestational weeks, 14 – 26 gestational weeks, and 27 gestational weeks until delivery), and for the whole
109 pregnancy. The starting date of the mother's last menstrual period together with ultrasound imaging were
110 used to estimate the date of conception (1).

NEXT GENERATION SEQUENCING

Total genomic DNA was isolated from buffy coats using the QIAamp DNA mini kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol. DNA concentrations were determined on a NanoDrop ND-1000 UV-Vis spectrometer (Thermo Scientific, Wilmington, DE) and kept at -80°C for future analyses.

Whole mitochondrial genome sequencing was performed by Macrogen Europe. Briefly, long range PCR on genomic DNA samples was performed to amplify the human mitochondrial genome. After quality control, library construction was carried out using the Nextera XT DNA Library Preparation Kit (Illumina) and samples were sequenced using the HiSeq X Ten platform (Illumina) with paired-end read length of 2 x 150 base pairs. To provide a quality control for cluster generation, sequencing, and alignment, a PhiX Spike (Illumina) of 1-2% was used. The demultiplexed fastq files were used for further analysis.

Downstream analyses of sequencing data (including alignments and variant calling) were performed with Geneious Prime (version 2021.2.2). Low-quality ends of sequences and low-quality regions, as well as all adapters (i.e., Nextera and PhiX) were trimmed using BBDuk. Next, sequences were aligned to the human mitochondrial reference genome (NC_012920, URL: https://www.ncbi.nlm.nih.gov/nuccore/NC_012920), after which variants were called. The Phred score was used to assess the base call accuracy. Heteroplasmy levels were evaluated based on the VAF. Only single nucleotide polymorphisms (SNPs) with a VAF > 5%, coverage > 300x, and a prevalence of at least 10% in the study population were included for further analysis, as previously recommended (26). In addition, six other variants were included, regardless of their prevalence, to define the most common European haplogroups (27).

MITOCHONDRIAL DNA CONTENT

Leukocyte mtDNA content was determined by assessing the ratio of the *MT-ND1* gene to the single copy nuclear *RPLP0* gene using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Details are available in supplemental information.

STATISTICAL ANALYSIS

Handling of the data and statistical analysis were performed using R (version 4.1.2) and RStudio software (version 2021.09.0). For descriptive analysis, categorical variables (i.e., neonate's gender, ethnicity, socioeconomic status, smoking status, parity, and season of delivery) were presented as numbers (frequency in %) and continuous variables (i.e., air pollution levels, gestational and maternal age, pre-pregnancy BMI, and mtDNA content) as means ± standard deviation (SD). 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. To determine the degree of correlation between the identified SNPs, principal component analysis was used (28). To allow for performing several comparisons while taking the correlation of the genomic data into account, we estimated the effective number of tests based on a principal component analysis. Seven components explained 90% of the variety of the data. Therefore, an adjusted significance level of $p = 0.05/7 = 0.007$ was applied.

In the first analysis, the association between *in utero* $PM_{2.5}$/NO_2 exposure and cord blood mitochondrial SNPs/haplogroups was analysed using multiple linear regression models corrected for *a priori* selected covariates based on previous literature (1, 29, 30): gestational age, newborn's sex, ethnicity, cord blood mtDNA content, maternal age, socioeconomic status, pre-pregnancy BMI, smoking during pregnancy, parity, and season of delivery. Cord blood mtDNA content was added to correct for possible variation in mtDNA input. These average exposure models were analysed for specific time-windows during pregnancy: first, second, and third trimester, and the whole pregnancy. Trimester-specific models were adjusted for one and each other. Mitochondrial heteroplasmy levels were evaluated based on the VAF (expressed as %). Therefore, estimates were provided as an additive change in VAF, with % as unit.

Based on the observed significant associations, the estimates for a 5 $\mu g/m^3$ increment in NO_2 were established at each gestational week using distributed lag linear models (DLMs) (31) to specify the sensitive time-window in detail. These models were adjusted for the above described covariates. Details on the

DLMs are available in the supplemental information. In sensitivity analysis, the association between cord blood mitochondrial SNPs and NO_2 was determined after excluding mothers who smoked during pregnancy. Week-specific estimates were provided as an additive change in VAF, with % as unit (i.e., heteroplasmy).

RESULTS

POPULATION CHARACTERISTICS

The general characteristics of the study population (n = 200) are provided in (Table 1~~Table 1~~). Gestation lasted on average 39.6 ± 1.4 weeks. The newborns weighed 3420 ± 424 g and most of them were girls (52%), predominantly of European descent (94%). Mothers were on average 29.7 ± 4.0 years old and had a pre-pregnancy BMI of 24.1 ± 4.5. The majority of the births was primiparous (53.5%) and took place between April 1st – September 30th (56.5%). 133 (66.5%) of the included mothers had a college or university degree and 24 (12%) mothers smoked during pregnancy. Cord blood mtDNA content averaged 1.1 ± 0.6. The residential prenatal air pollution exposures by gestational time window are provided in Table 2~~Table 2~~. The average ambient $PM_{2.5}$ and NO_2 concentrations during the entire pregnancy were 15.5 ± 2.1 and 20.2 ± 4.5 µg/m³, respectively. The correlations of $PM_{2.5}$ and NO_2 concentrations across the three trimesters of pregnancy are presented in **Supplementary Table S1**. Strong correlations are found between the $PM_{2.5}$ concentrations of the first and third trimester and the NO_2 concentrations of the second and third trimester of pregnancy.

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

Characteristic		**Characteristic**	
Maternal	Mean ± SD or *n* (%)	*Newborn*	Mean ± SD or *n* (%)
Age at delivery (years)	29.7 ± 4.0	Gestational age (weeks)	39.6 ± 1.4

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Pre-pregnancy BMI	24.1 ± 4.5	Birth weight (g)	3420 ± 424
Smoking during pregnancy (yes)	24 (12.0)	Sex (female)	104 (52.0)
Parity		Ethnicity	
Primiparous	107 (53.5)	European	188 (94.0)
Secundiparous	69 (34.5)	Non-European	12 (6.0)
Multiparous	24 (12.0)		
Socioeconomic status			
Low	12 (6.0)		
Middle	55 (27.5)		
High	133 (66.5)		
Season of delivery			
Cold (October 1st – March 31st)	87 (43.5)		
Warm (April 1st – September 30th)	113 (56.5)		

Table 2: Residential prenatal exposure concentrations of $PM_{2.5}$ and NO_2 (in µg/m³) by gestational time window (n = 200).

	Mean ± SD	25th percentile	75th percentile
$PM_{2.5}$			
Trimester 1	14.6 ± 5.4	10.2	18.2
Trimester 2	16.6 ± 5.1	12.1	20.6
Trimester 3	15.3 ± 5.9	10.1	19.7
Whole pregnancy	15.5 ± 2.1	14.1	16.8
NO_2			
Trimester 1	20.0 ± 6.3	15.1	24.4
Trimester 2	21.0 ± 6.1	16.8	25.1
Trimester 3	19.6 ± 6.4	14.5	23.6
Whole pregnancy	20.2 ± 4.5	17.0	22.8

IDENTIFICATION OF CORD BLOOD MITOCHONDRIAL MUTATIONS

A total of 2,928 cord blood mtDNA variants were identified in our population (**Supplementary Figure S2**), of which 28 mtDNA SNPs were included for the analysis (Table 3~~Table 3~~). Haplogroups I, J, V, W, X were (almost) not present in our population and were not included in further analyses (**Supplementary Table S2**). The average level of heteroplasmy of all identified variants in our study population was 95.2%, ranging from 5% to 100%. The mean SNP coverage was 19,627x, ranging from 3014x to 57719x. The average quality score (i.e., Phred Score) was 39 (range: 36 to 40), indicating an average base call accuracy of 99.87%.

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Table 3: Identified cord blood mitochondrial mutations. *Variants included to define European haplogroups, regardless of their prevalence in the study population. CDS: Coding DNA Sequence.

	Reference SNP	CDS	Prevalence (%) in study population (n = 200)
D-Loop			
MT-D-Loop$_{310T>C}$	-	No	72 (36.0)
MT-D-Loop$_{489T>C}$	-	No	23 (11.5)
MT-D-Loop$_{16126T>C}$	-	No	27 (13.5)
MT-D-Loop$_{16189T>C}$	-	No	23 (11.5)
MT-D-Loop$_{16311T>C}$	-	No	25 (12.5)
MT-D-Loop$_{16391G>A}$*	-	No	2 (1.0)
MT-D-Loop$_{16519T>C}$	-	No	58 (29.0)
Mitochondrially Encoded 16S RRNA (*MT-RNR2*)			
MT-RNR2$_{1719G>A}$*	rs3928305	No	-
MT-RNR2$_{1888G>A}$	rs2897260	No	21 (10.5)
MT-RNR2$_{2706A>G}$	rs2854128	No	116 (58.0)
MT-RNR2$_{3010G>A}$	rs3928306	No	50 (25.0)
Mitochondrially Encoded NADH Dehydrogenase 1 (*MT-ND1*)			
MT-ND1$_{4216T>C}$	rs1599988	Yes	43 (21.5)

Mitochondrially Encoded NADH Dehydrogenase 2 (*MT-ND2*)			
MT-ND2$_{4580G>A}$*	rs28357975	Yes	2 (1.0)
Mitochondrially Encoded Cytochrome C Oxidase I (*MT-COX1*)			
MT-COX1$_{7028C>T}$	rs2015062	Yes	115 (57.5)
Mitochondrially Encoded Cytochrome C Oxidase II (*MT-COX2*)			
MT-COX2$_{8251G>A}$*	rs3021089	Yes	2 (1.0)
Mitochondrially Encoded ATP Synthase Membrane Subunit 6 (*MT-ATP6*)			
MT-ATP6$_{8697G>A}$	rs879233543	Yes	20 (10.0)
MT-ATP6$_{8860A>G}$	rs2001031	Yes	168 (84.0)
MT-ATP6$_{9055G>A}$*	rs193303045	Yes	11 (5.5)
Mitochondrially Encoded TRNA-Arg (*MT-TRNR*)			
MT-TRNR$_{10463T>C}$	rs28358279	No	24 (12.0)
Mitochondrially Encoded NADH Dehydrogenase 3 (*MT-ND3*)			
MT-ND3$_{10398A>G}$	rs2853826	Yes	34 (17.0)
Mitochondrially Encoded NADH Dehydrogenase 4 (*MT-ND4*)			
MT-ND4$_{11251A>G}$	rs869096886	Yes	22 (11.0)
MT-ND4$_{11467A>G}$	rs2853493	Yes	33 (16.5)
MT-ND4$_{11719G>A}$	rs2853495	Yes	101 (50.5)
MT-ND4$_{11812A>G}$	rs3088053	Yes	20 (10.0)
Mitochondrially Encoded TRNA-Leu (CUN) 2 (*MT-TRNL2*)			
MT-TRNL2$_{12308A>G}$	rs2853498	No	31 (15.5)
Mitochondrially Encoded NADH Dehydrogenase 5 (*MT-ND5*)			
MT-ND5$_{12372G>A}$	rs2853499	Yes	33 (16.5)
MT-ND5$_{12612A>G}$	rs28359172	Yes	26 (13.0)
MT-ND5$_{13368G>A}$	rs3899498	Yes	21 (10.5)
MT-ND5$_{13708G>A}$*	rs28359178	Yes	-
Mitochondrially Encoded NADH Dehydrogenase 6 (*MT-ND6*)			
MT-ND6$_{14233A>G}$	rs3915611	Yes	20 (10.0)
Mitochondrially Encoded Cytochrome B (*CYTB*)			
MT-CYTB$_{14766C>T}$	rs193302980	Yes	58 (29.0)
MT-CYTB$_{14798T>C}$	rs28357681	Yes	21 (10.5)

MT-CYTB$_{15452C>A}$	rs193302994	Yes	40 (20.0)
MT-CYTB$_{15607A>G}$	rs193302996	Yes	21 (10.5)

ASSOCIATION OF MITOCHONDRIAL HETEROPLASMY IN CORD BLOOD WITH PRENATAL AIR POLLUTION

In total, the association of 28 cord blood mtDNA variants and four haplogroups with prenatal $PM_{2.5}$ and NO_2 exposure was explored using multiple linear models for the whole pregnancy and each trimester separately. These models were adjusted for preselected covariates. The association between prenatal NO_2 exposure and cord blood mtDNA variants was significant for two identified SNPs (Figure 1~~Figure 1~~). Cord blood MT-D-Loop$_{16311T>C}$ was negatively associated with *in utero* exposure to NO_2 in the second trimester of pregnancy (-2.06%; 95% CI: -3.54 to -0.57; p = 0.007), while cord blood MT-CYTB$_{14766C>T}$ was positively associated with prenatal NO_2 exposure in the third trimester of pregnancy (2.74%; 95% CI: 0.93 to 4.54; p = 0.003). No significant associations were observed with prenatal $PM_{2.5}$ exposure (Figure 1~~Figure 1~~), nor with mitochondrial haplogroups and prenatal air pollution (data not shown). The overall heteroplasmy load was not significantly linked with *in utero* $PM_{2.5}$ exposure, but prenatal NO_2 exposure was borderline significantly associated with overall heteroplasmy load in the second trimester of pregnancy (-0.30%; 95% CI: -0.65 to 0.04; p = 0.08).

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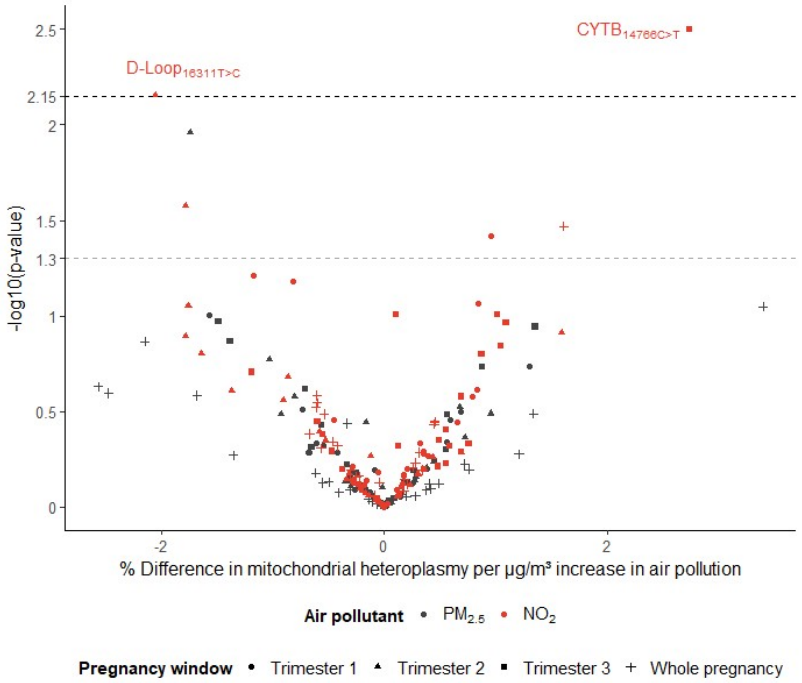

Figure 1: Volcano plot showing the association between $PM_{2.5}$ (grey) and NO_2 (red) and all identified cord blood mitochondrial SNPs. Two SNPs were significantly associated with prenatal NO_2 exposure ($p < 0.007$; black dotted line). Models were adjusted for gestational age, newborn's sex, cord blood mtDNA content, ethnicity, maternal age, socioeconomic status, pre-pregnancy BMI, smoking during pregnancy, parity, and season of delivery. Estimates were provided as % difference in VAF per µg/m³ increase in $PM_{2.5}$ or NO_2 for the first trimester (●), second trimester (▲), third trimester (■), and the whole pregnancy (+). The grey dotted line represents $p = 0.05$.

For cord blood MT-D-Loop$_{16311T>C}$, the week-specific DLM model showed a negative association with NO_2 exposure in mid pregnancy. A 5 µg/m³ increment in NO_2 was linked with cord blood MT-D-Loop$_{16311T>C}$ heteroplasmy from gestational week 17 – 25, with the largest effect in week 20 (-0.87%; 95% CI: -1.62 to -0.12; p = 0.02; **Figure 2~~Figure 2~~A, Supplementary Table S3**). Cord blood MT-CYTB$_{14766C>T}$ was negatively associated with NO_2 exposure in mid pregnancy, from gestational week 14 – 17, and positively associated in late pregnancy, from gestational week 31 – 36. In the third trimester, week 35 had the largest effect (1.18%; 95% CI: 0.28 to 2.08; p = 0.01; **Figure 2~~Figure 2~~B, Supplementary Table S3**). In sensitivity analyses, excluding mothers who smoked during pregnancy, similar trends were observed. Cord blood MT-D-Loop$_{16311T>C}$ heteroplasmy was negatively associated with NO_2 exposure from gestational week 23 – 24, with the largest effect in week 23 (-0.68%; 95% CI: -1.31 to -0.04; p = 0.04; **Supplementary Table S4**). Compared with the main analysis, cord blood MT-CYTB$_{14766C>T}$ was only still positively associated with NO_2 exposure in late pregnancy, from gestational week 34 – 36, with the largest effect in week 36 (1.12%; 95% CI: 0.01 to 2.23; p = 0.049; **Supplementary Table S4**). The sample size was reduced from n = 200 to n = 176. In addition, when excluding infants of non-European ethnicity, cord blood MT-D-Loop$_{16311T>C}$ heteroplasmy was negatively associated with NO_2 exposure from gestational week 17 – 25, with the largest effect in week 21 (-0.92%; 95% CI: -1.66 to -0.18; p = 0.02; **Supplementary Table S5**). Cord blood MT-CYTB$_{14766C>T}$ heteroplasmy was positively linked with NO_2 exposure from gestational week 32 – 37, with the largest effect in week 37 (1.35%; 95% CI: 0.01 to 2.69; p = 0.049; **Supplementary Table S5**), after excluding non-European infants. Lastly, when excluding mothers diagnosed with gestational diabetes mellitus and hypertension, cord blood MT-D-Loop$_{16311T>C}$ heteroplasmy was negatively associated with NO_2 exposure from gestational week 18 – 25, with the largest effect in week 21 (-0.92%; 95% CI: -1.70 to -0.14; p = 0.02; **Supplementary Table S6**). Furthermore, cord blood MT-CYTB$_{14766C>T}$ heteroplasmy was negatively associated with NO_2 exposure from gestational week 14 – 16, while it was positively linked from gestational

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week 32 – 36, with the largest effect in week 36 (1.35%; 95% CI: 0.22 to 2.49; p = 0.02; **Supplementary Table S6**).

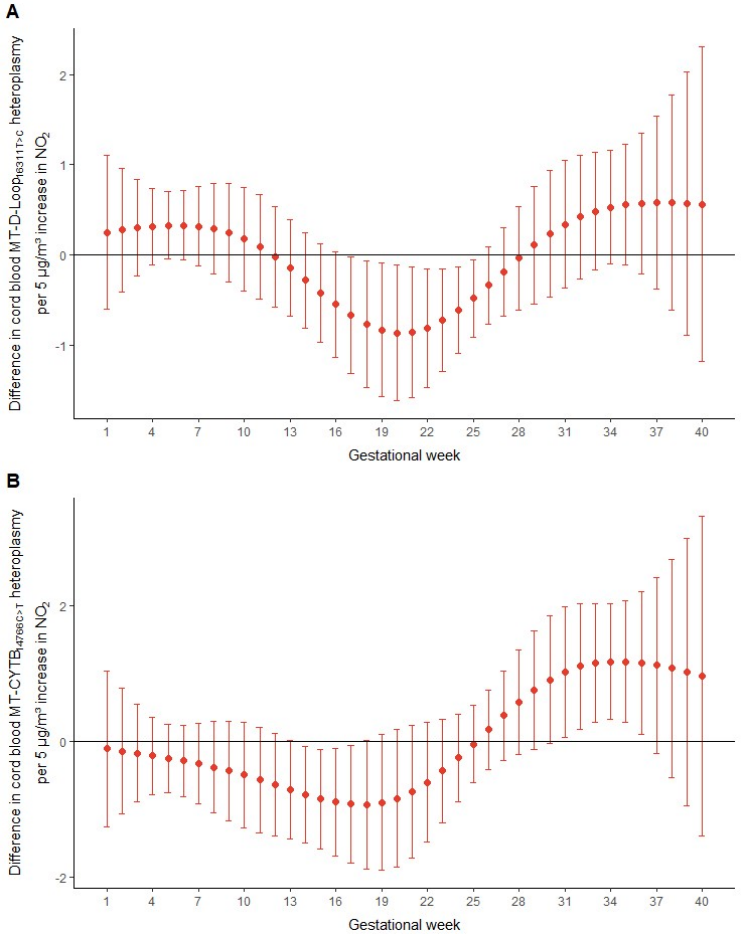

Figure 2: Difference in cord blood mitochondrial heteroplasmy in association with week-specific prenatal exposures to NO_2 for (A) MT-D-Loop$_{16311T>C}$ and (B) MT-CYTB$_{14766C>T}$ (n = 200). Week-specific estimates are given as

% difference in VAF per 5 µg/m³ increment in NO_2. Models were adjusted for gestational age, newborn's sex, cord blood mtDNA content, ethnicity, maternal age, socioeconomic status, pre-pregnancy BMI, smoking during pregnancy, parity, and season of delivery. Error bars stand for the 95% CI for each weekly estimate.

DISCUSSION

MtDNA heteroplasmy is remarkably common and providing new perspectives on the etiology of complex diseases. It might also be relevant in a better understanding of the concept of Developmental Origin of Health and Diseases (DOHaD). In this study, we observed that *in utero* exposure to NO_2 is linked with cord blood mtDNA heteroplasmy of MT-D-Loop$_{16311T>C}$ and MT-CYTB$_{14766C>T}$. Prenatal NO_2 was negatively associated with cord blood MT-D-Loop$_{16311T>C}$, from gestational week 17 – 25, and with cord blood MT-CYTB$_{14766C>T}$, from gestational week 14 – 17. In addition, NO_2 exposure during pregnancy was positively associated with cord blood MT-CYTB$_{14766C>T}$, from gestational week 31 – 36. No significant associations were observed with prenatal $PM_{2.5}$ exposure.

Mitochondria are sensitive to damage induced by oxidative stress, which can be generated by various air pollutants, including polycyclic aromatic hydrocarbons (PAHs), carbon monoxide (CO), volatile organic compounds (VOCs), NO_2, and PM (32). Both prenatal $PM_{2.5}$ (33) and NO_2 (16) exposure were linked with lower mtDNA content, an indicator of mitochondrial (dys)function, in cord blood and placental tissue, respectively. In contrast, little evidence is available linking prenatal air pollution to mtDNA mutations. *In utero* exposure to $PM_{2.5}$ was linked with the cord blood MT-ND4L$_{10550A>G}$ variant in newborns (34). In addition, another study observed an association between placental mtDNA mutational load and prenatal $PM_{2.5}$ exposure. These mutations were predominantly found in MT-ND5, MT-ATP6, and MT-ATP8 (13). The association between *in utero* air pollution exposure and these mtDNA mutations were mainly observed in mid- to late-pregnancy, which is consistent with our results. Our study showed a similar borderline trend with mutational load in cord blood and prenatal NO_2 exposure in the second trimester of pregnancy.

We identified that MT-D-Loop$_{16311T>C}$ heteroplasmy was associated with prenatal NO_2 exposure. The mtDNA contains a hypervariable, non-coding displacement loop, also known as the D-Loop, which acts as a promoter for both the heavy and light strands of the mtDNA and harbors most of the regulatory sequences related to mtDNA replication, transcription, and translation (35-37). This D-Loop contains three hypervariable regions: HV1 (positions 16024 – 16383), HV2 (positions 57 – 372), and HV3 (positions 438 – 574) (38). MT-D-Loop$_{16311T>C}$ is a SNP in HV1, which was also the most common SNP identified in a sequence analysis of hypervariable regions in ten unrelated Iranian families (39). Several studies linked mutations in the mitochondrial D-Loop with cancer (37, 40, 41). As the D-Loop region regulates the replication, transcription, and translation of mtDNA, mutations in this region might cause impaired mitochondrial function due to the abnormal expression of mitochondrial proteins (35, 36, 42). Secondly, prenatal NO_2 exposure was linked with MT-CYTB$_{14766C>T}$. The *MT-CYTB* gene encodes one of the proteins of complex III, which plays a role in oxidative phosphorylation. Mutations in this gene can cause mitochondrial complex III deficiency, which is characterized by muscle weakness and pain. Individuals with higher heteroplasmy levels of these mutations can also encounter problems with their liver, kidneys, heart, and brain (43). The mutation MT-CYTB$_{14766C>T}$ induces a codon change from ACT>ATT, causing a change in amino acid from threonine to isoleucine, classifying it as a nonsynonymous SNP (44). One study reported that MT-CYTB$_{14766C>T}$ was significantly different in patients with normal-tension glaucoma, compared with controls (45). ClinVar, a database of the relationships between human genetic variants and diseases (46), reported a link between MT-CYTB$_{14766C>T}$ and familial breast cancer (47) and Leigh syndrome (48). However, no scientific reference was available for these conditions. Based on literature, none of the 28 included SNPs were reported as highly pathogenic. Only one (i.e., rs267606617) out of ten pathogenic mtDNA variants often found in mitochondrial disease (49) was identified once in our study population, which is linked with maternally transmitted non-syndromic deafness (50).

More research concerning the identified SNPs in our study is warranted. Several studies reported associations between other mitochondrial SNPs and health disorders, such as obesity (51), BMI (52), body fat mass (53), blood pressure and fasting blood glucose levels (54), and MELAS (55). A mitochondrial genome-wide association study (GWAS) identified ten SNPs that were associated with HDL cholesterol and one SNP with triglycerides levels (56). Particularly individuals with the highest levels of heteroplasmy of MT-TRNL1$_{3243A>G}$ had a higher risk of all-cause, dementia, and stroke mortality (57). Also, in cancer development, studies reported a heteroplasmy-shifting phenomenon as a potential biomarker of tumour progression and treatment response (58). For example, changes in heteroplasmy levels were observed in MT-COX1$_{6419A}$, which was heteroplasmic in normal samples while homoplasmic in tumour samples (59).

This is the first study to show that prenatal NO_2 exposure is associated with cord blood mitochondrial mutations and suggests two critical windows of exposure in mid- to late-pregnancy. It has already been reported that the placenta and mitochondria establish structural changes during the gestational period (60). Initially in the first trimester, the embryo develops in a low oxygen environment, promoting organogenesis, while in the third trimester, the rapid growth of the foetus demands higher oxygen levels (61). Between gestational week 10 – 12, the maternal blood flow to the placenta is established (62) and around the same time, mitochondrial respiration and placental mtDNA content increased (60). These changes may indicate an increase in mitochondrial biogenesis, which in turn results in enhanced antioxidant defences (60, 63). Mitochondrial biogenesis may be initiated as a response to adverse conditions, such as preeclampsia or intrauterine growth restriction (IUGR), as these conditions were linked with an increase in placental mtDNA content (64). A possible explanation of the observed negative associations between prenatal NO_2 exposure and MT-D-Loop$_{16311T>C}$ and MT-CYTB$_{14766C>T}$ in the second trimester of pregnancy might be an increase in mitochondrial biogenesis, resulting in improved antioxidant defences. In contrast, MT-CYTB$_{14766C>T}$ was positively associated with *in utero* exposure to NO_2 in the third trimester, which can be explained by the fact that oxidative stress increased with gestational age (62).

Also, mitochondrial respiration was lower at term placenta's, compared to early in pregnancy (60), suggesting mitochondrial deficiency, possibly making them more susceptible for oxidative damage.

Strengths and limitations – Our study has several strengths. To assess the daily air pollution exposures during the entire pregnancy, we used a validated, high-resolution spatial-temporal interpolation method that has been used in multiple studies (65-67). We used whole mitochondrial genome sequencing to analyse the entire mitochondrial genome. As the mean coverage was 19,627x, base calls were made with a high degree of confidence. Despite these strengths, we acknowledge some potential limitations in our study. Other environmental elements may influence mtDNA mutations. However, our statistical models were adjusted for multiple confounders, including sociodemographic and environmental factors (e.g., season of delivery), which are related to diverse exposures (13). Air pollution exposures were modelled purely on the maternal residential address, not considering other sources of exposure. Nevertheless, the modelled air pollution exposures have shown to reflect the accumulation of particles in the placenta (68), suggesting a proper estimation of an individual's exposures.

SUPPORTING INFORMATION

Study population. Prenatal air pollution exposure assessment. Measurement of mtDNA content. Distributed lag linear models. Supplementary Table S1: Correlations pollutants across trimesters. **Supplementary Table S2**: Defined European haplogroups. **Supplementary Table S3**: Estimates main analysis. **Supplementary Table S4**: Estimates sensitivity analysis excluding smokers. **Supplementary Table S5**: Estimates sensitivity analysis excluding non-European ethnicity. **Supplementary Table S6**: Estimates sensitivity analysis excluding gestational diabetes mellitus and hypertension. **Supplementary Figure S1**: Flowchart study population. **Supplementary Figure S2**: All identified mitochondrial variants.

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AUTHOR CONTRIBUTIONS

TSN and MP coordinate the ENVIR*ON*AGE birth cohort. MP and CC designed the research hypothesis. CV provided air pollution exposure data. CC performed the experiments. CC, CW, and MP analysed the data and interpreted the results. CC drafted the article. KS, DSM, and WL critically reviewed the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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