

In Utero Exposure to Air Pollutants and Mitochondrial Heteroplasmy in Neonates

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1 *In utero* exposure to air pollutants and mitochondrial heteroplasmy in
2 neonates

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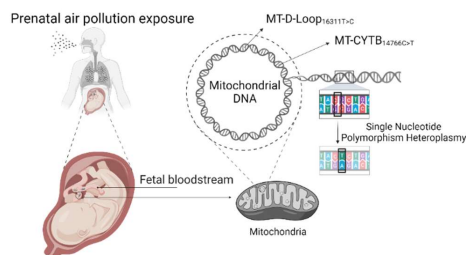
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12 **ABSTRACT ART**

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16 **ABSTRACT**

17 Mitochondria are sensitive to oxidative stress, which can be caused by traffic-related air pollution.
18 Placental mtDNA mutations have been previously linked with air pollution. However, the relationship
19 between prenatal air pollution and cord blood mtDNA mutations has been poorly understood. Therefore,
20 we hypothesized that prenatal PM_{2.5} and NO₂ exposures are associated with cord blood mtDNA
21 heteroplasmy. As part of the ENVIRONAGE cohort, 200 mother-newborn pairs were recruited. Cord blood
22 mitochondrial SNPs were identified by whole mitochondrial genome sequencing and heteroplasmy levels

23 were evaluated based on the variant allele frequency (VAF). Outdoor PM_{2.5} and NO₂ concentrations were
24 determined by a high-resolution spatial-temporal interpolation method, based on the maternal residential
25 address. Distributed lag linear models (DLMs) were used to determine sensitive time windows for the
26 association between NO₂ exposure and cord blood mtDNA heteroplasmy. A 5 µg/m³ increment in NO₂ was
27 linked with MT-D-Loop_{16311T>C} heteroplasmy from gestational week 17–25. MT-CYTB_{14766C>T} was negatively
28 associated with NO₂ exposure in mid pregnancy, from week 14–17, and positively associated in late
29 pregnancy, from week 31–36. No significant associations were observed with prenatal PM_{2.5} exposure.
30 This is the first study to show that prenatal NO₂ exposure is associated with cord blood mitochondrial
31 mutations and suggests two critical windows of exposure in mid- to late-pregnancy.

32 SYNOPSIS

33 Prenatal NO₂ exposure is associated with cord blood mitochondrial D-Loop_{16311T>C} and CYTB_{14766C>T}
34 heteroplasmy in mid- to late-pregnancy.

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

36 INTRODUCTION

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

46 8, 9). Several studies showed that higher levels of heteroplasmy were related to several diseases (8-10). A
47 minimum number of mutated mtDNA sequences is necessary before mitochondrial dysfunction will
48 appear, which is the so-called threshold effect. This threshold ranged between 60-90% mutant to wild type
49 mtDNA, dependent on the mutation and tissue (11). Heteroplasmy levels can accumulate, leading to
50 impaired mitochondrial function, affecting human health (12). One study reported that the mutational
51 load in mtDNA of placental tissue, in particular in genes coding NADH dehydrogenase and subunits of ATP
52 synthase, was associated with prenatal exposure to fine particulate matter (PM_{2.5}) (13).

53 Mitochondria are sensitive to damage by oxidative stress, which can be caused by traffic-related air
54 pollution such as PM_{2.5} and nitrogen dioxide (NO₂) (14). PM_{2.5} exposure was significantly associated with
55 mitochondrial 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels, a biomarker for oxidative stress, in maternal
56 blood during the entire pregnancy (15). In addition, a 10 µg/m³ increment in NO₂ exposure during
57 pregnancy was associated with a 4.9% decrease in placental mtDNA content (16). Prenatal NO₂ exposure
58 also inversely affected infant growth parameters, such as height at six months of age, which could be
59 mediated by placental mtDNA content (17). Furthermore, NO₂ exposure in rats impaired the mitochondrial
60 energy metabolism, including reduced ATP production and increased reactive oxygen species (ROS)
61 production, as well as inhibition of mitochondrial biogenesis (18). Since there has been a considerable
62 increase of evidence reporting the influence of air pollution on human health, the World Health
63 Organization (WHO) updated their air quality guidelines (AQG) in September 2021 (19). The European
64 Environment Agency (EEA) monitors PM_{2.5} levels in 340 European cities (20). Based on the measurements
65 of 2020 and 2021, the PM_{2.5} concentrations in Flanders, Belgium ranged from 10.4 to 12.2 µg/m³, which is
66 classified as moderate air quality (WHO AQG). The Belgian annual mean NO₂ level in 2019 respected the
67 threshold of the EU (i.e., Air Quality Directive 2008/50/EC), but was almost double of the NO₂ WHO
68 guidelines (21).

69 Although the association between air pollution and mitochondria has been studied before, the role of
70 prenatal air pollution exposure on cord blood mitochondrial mutations is poorly understood. As cord blood
71 reflects the state of the foetus and its mitochondrial makeup at the start of life (13), having insight into
72 this mechanism can unravel complex mechanisms of disease onset related to mitochondrial function, such
73 as neurodegenerative disorders (22, 23), and thereby contribute to the “Developmental Origins of Health
74 and Disease” hypothesis (DOHaD) that states that health or diseases may find their origin in early life (24).
75 Therefore, we hypothesized that *in utero* exposure to PM_{2.5} and NO₂ is linked with mitochondrial
76 heteroplasmy in cord blood of newborns.

77 MATERIALS AND METHODS

78 STUDY POPULATION

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

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

86 DATA AND SAMPLE COLLECTION

87 Immediately after delivery, umbilical cord blood was sampled in Vacutainer® Plus Plastic K2EDTA Tubes
88 (BD, Franklin Lakes, NJ, USA) and centrifuged for 15 min at 3200 rpm to retrieve buffy coats containing
89 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/m²) 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 NO₂ concentrations (in µg/m³), 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).

111 NEXT GENERATION SEQUENCING

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

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

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

130 MITOCHONDRIAL DNA CONTENT

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

134 STATISTICAL ANALYSIS

135 Handling of the data and statistical analysis were performed using R (version 4.1.2) and RStudio software
136 (version 2021.09.0). For descriptive analysis, categorical variables (i.e., neonate's gender, ethnicity,
137 socioeconomic status, smoking status, parity, and season of delivery) were presented as numbers
138 (frequency in %) and continuous variables (i.e., air pollution levels, gestational and maternal age, pre-
139 pregnancy BMI, and mtDNA content) as means \pm standard deviation (SD). The residuals of the regression
140 models, as well as a visual inspection of histograms and QQ-plots were used to evaluate the normality
141 assumption and they did not deviate from normality. To determine the degree of correlation between the
142 identified SNPs, principal component analysis was used (28). To allow for performing several comparisons
143 while taking the correlation of the genomic data into account, we estimated the effective number of tests
144 based on a principal component analysis. Seven components explained 90% of the variety of the data.
145 Therefore, an adjusted significance level of $p = 0.05/7 = 0.007$ was applied.

146 In the first analysis, the association between *in utero* PM_{2.5}/NO₂ exposure and cord blood mitochondrial
147 SNPs/haplogroups was analysed using multiple linear regression models corrected for *a priori* selected
148 covariates based on previous literature (1, 29, 30): gestational age, newborn's sex, ethnicity, cord blood
149 mtDNA content, maternal age, socioeconomic status, pre-pregnancy BMI, smoking during pregnancy,
150 parity, and season of delivery. Cord blood mtDNA content was added to correct for possible variation in
151 mtDNA input. These average exposure models were analysed for specific time-windows during pregnancy:
152 first, second, and third trimester, and the whole pregnancy. Trimester-specific models were adjusted for
153 one and each other. Mitochondrial heteroplasmy levels were evaluated based on the VAF (expressed as
154 %). Therefore, estimates were provided as an additive change in VAF, with % as unit.

155 Based on the observed significant associations, the estimates for a 5 $\mu\text{g}/\text{m}^3$ increment in NO₂ were
156 established at each gestational week using distributed lag linear models (DLMs) (31) to specify the sensitive
157 time-window in detail. These models were adjusted for the above described covariates. Details on the

158 DLMS are available in the supplemental information. In sensitivity analysis, the association between cord
 159 blood mitochondrial SNPs and NO₂ was determined after excluding mothers who smoked during
 160 pregnancy. Week-specific estimates were provided as an additive change in VAF, with % as unit (i.e.,
 161 heteroplasmy).

162 **RESULTS**

163 POPULATION CHARACTERISTICS

164 The general characteristics of the study population (n = 200) are provided in [\(Table 1Table-1\)](#). Gestation
 165 lasted on average 39.6 ± 1.4 weeks. The newborns weighed 3420 ± 424 g and most of them were girls
 166 (52%), predominantly of European descent (94%). Mothers were on average 29.7 ± 4.0 years old and had
 167 a pre-pregnancy BMI of 24.1 ± 4.5. The majority of the births was primiparous (53.5%) and took place
 168 between April 1st – September 30th (56.5%). 133 (66.5%) of the included mothers had a college or
 169 university degree and 24 (12%) mothers smoked during pregnancy. Cord blood mtDNA content averaged
 170 1.1 ± 0.6. The residential prenatal air pollution exposures by gestational time window are provided in [Table](#)
 171 [2Table-2](#). The average ambient PM_{2.5} and NO₂ concentrations during the entire pregnancy were 15.5 ± 2.1
 172 and 20.2 ± 4.5 µg/m³, respectively. The correlations of PM_{2.5} and NO₂ concentrations across the three
 173 trimesters of pregnancy are presented in **Supplementary Table S1**. Strong correlations are found between
 174 the PM_{2.5} concentrations of the first and third trimester and the NO₂ concentrations of the second and
 175 third trimester of pregnancy.

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

Characteristic		Characteristic	
<i>Maternal</i>	Mean ± SD or n (%)	<i>Newborn</i>	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 1 st – March 31 st)	87 (43.5)		
Warm (April 1 st – September 30 th)	113 (56.5)		

177

178 **Table 2: Residential prenatal exposure concentrations of PM_{2.5} and NO₂ (in µg/m³) by gestational time window (n**
179 **= 200).**

	Mean ± SD	25 th percentile	75 th 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
NO₂			
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

180

181 IDENTIFICATION OF CORD BLOOD MITOCHONDRIAL MUTATIONS

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

188
 189 **Table 3: Identified cord blood mitochondrial mutations.** *Variants included to define European haplogroups,
 190 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 (<i>MT-RNR2</i>)			
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 (<i>MT-ND1</i>)			
MT-ND1 _{4216T>C}	rs1599988	Yes	43 (21.5)

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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)

191
 192 ASSOCIATION OF MITOCHONDRIAL HETEROPLASMY IN CORD BLOOD WITH PRENATAL AIR
 193 POLLUTION

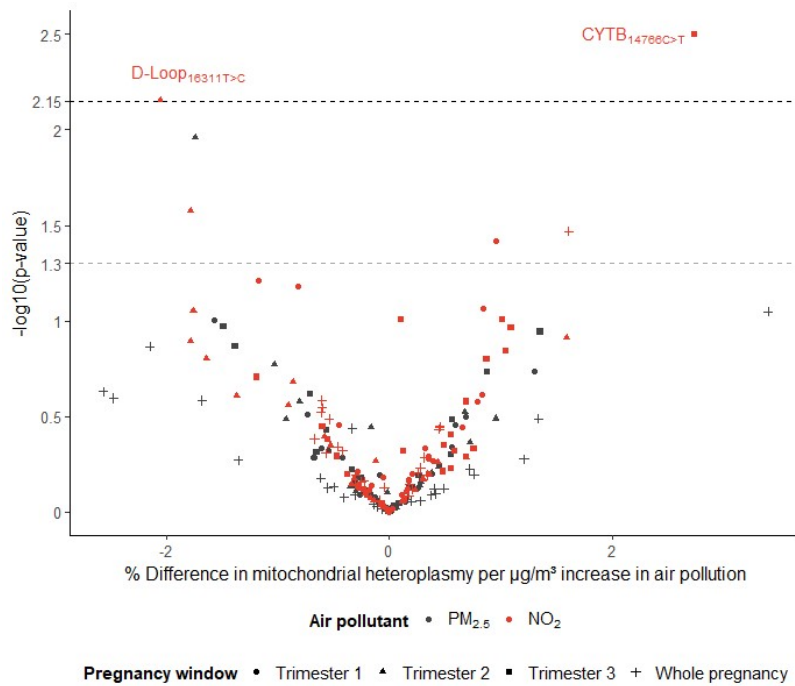
194 In total, the association of 28 cord blood mtDNA variants and four haplogroups with prenatal PM_{2.5} and
 195 NO₂ exposure was explored using multiple linear models for the whole pregnancy and each trimester
 196 separately. These models were adjusted for preselected covariates. The association between prenatal NO₂
 197 exposure and cord blood mtDNA variants was significant for two identified SNPs (Figure 1~~Figure 1~~). Cord
 198 blood MT-D-Loop_{16311T>C} was negatively associated with *in utero* exposure to NO₂ in the second trimester
 199 of pregnancy (-2.06%; 95% CI: -3.54 to -0.57; p = 0.007), while cord blood MT-CYTB_{14766C>T} was positively
 200 associated with prenatal NO₂ exposure in the third trimester of pregnancy (2.74%; 95% CI: 0.93 to 4.54; p
 201 = 0.003). No significant associations were observed with prenatal PM_{2.5} exposure (Figure 1~~Figure 1~~), nor
 202 with mitochondrial haplogroups and prenatal air pollution (data not shown). The overall heteroplasmy
 203 load was not significantly linked with *in utero* PM_{2.5} exposure, but prenatal NO₂ exposure was borderline
 204 significantly associated with overall heteroplasmy load in the second trimester of pregnancy (-0.30%; 95%
 205 CI: -0.65 to 0.04; p = 0.08).

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 207 **Figure 1: Volcano plot showing the association between PM_{2.5} (grey) and NO₂ (red) and all identified cord blood**
 208 **mitochondrial SNPs.** Two SNPs were significantly associated with prenatal NO₂ exposure ($p < 0.007$; black dotted
 209 line). Models were adjusted for gestational age, newborn's sex, cord blood mtDNA content, ethnicity, maternal age,
 210 socioeconomic status, pre-pregnancy BMI, smoking during pregnancy, parity, and season of delivery. Estimates were
 211 provided as % difference in VAF per $\mu\text{g}/\text{m}^3$ increase in PM_{2.5} or NO₂ for the first trimester (\bullet), second trimester (\blacktriangle),
 212 third trimester (\blacksquare), and the whole pregnancy ($+$). The grey dotted line represents $p = 0.05$.

213 For cord blood MT-D-Loop_{16311T>C}, the week-specific DLM model showed a negative association with NO₂
214 exposure in mid pregnancy. A 5 µg/m³ increment in NO₂ was linked with cord blood MT-D-Loop_{16311T>C}
215 heteroplasmy from gestational week 17 – 25, with the largest effect in week 20 (-0.87%; 95% CI: -1.62 to -
216 0.12; p = 0.02; **Figure 2Figure-2A, Supplementary Table S3**). Cord blood MT-CYTB_{14766C>T} was negatively
217 associated with NO₂ exposure in mid pregnancy, from gestational week 14 – 17, and positively associated
218 in late pregnancy, from gestational week 31 – 36. In the third trimester, week 35 had the largest effect
219 (1.18%; 95% CI: 0.28 to 2.08; p = 0.01; **Figure 2Figure-2B, Supplementary Table S3**). In sensitivity analyses,
220 excluding mothers who smoked during pregnancy, similar trends were observed. Cord blood MT-D-
221 Loop_{16311T>C} heteroplasmy was negatively associated with NO₂ exposure from gestational week 23 – 24,
222 with the largest effect in week 23 (-0.68%; 95% CI: -1.31 to -0.04; p = 0.04; **Supplementary Table S4**).
223 Compared with the main analysis, cord blood MT-CYTB_{14766C>T} was only still positively associated with NO₂
224 exposure in late pregnancy, from gestational week 34 – 36, with the largest effect in week 36 (1.12%; 95%
225 CI: 0.01 to 2.23; p = 0.049; **Supplementary Table S4**). The sample size was reduced from n = 200 to n =
226 176. In addition, when excluding infants of non-European ethnicity, cord blood MT-D-Loop_{16311T>C}
227 heteroplasmy was negatively associated with NO₂ exposure from gestational week 17 – 25, with the largest
228 effect in week 21 (-0.92%; 95% CI: -1.66 to -0.18; p = 0.02; **Supplementary Table S5**). Cord blood MT-
229 CYTB_{14766C>T} heteroplasmy was positively linked with NO₂ exposure from gestational week 32 – 37, with
230 the largest effect in week 37 (1.35%; 95% CI: 0.01 to 2.69; p = 0.049; **Supplementary Table S5**), after
231 excluding non-European infants. Lastly, when excluding mothers diagnosed with gestational diabetes
232 mellitus and hypertension, cord blood MT-D-Loop_{16311T>C} heteroplasmy was negatively associated with NO₂
233 exposure from gestational week 18 – 25, with the largest effect in week 21 (-0.92%; 95% CI: -1.70 to -0.14;
234 p = 0.02; **Supplementary Table S6**). Furthermore, cord blood MT-CYTB_{14766C>T} heteroplasmy was negatively
235 associated with NO₂ exposure from gestational week 14 – 16, while it was positively linked from gestational

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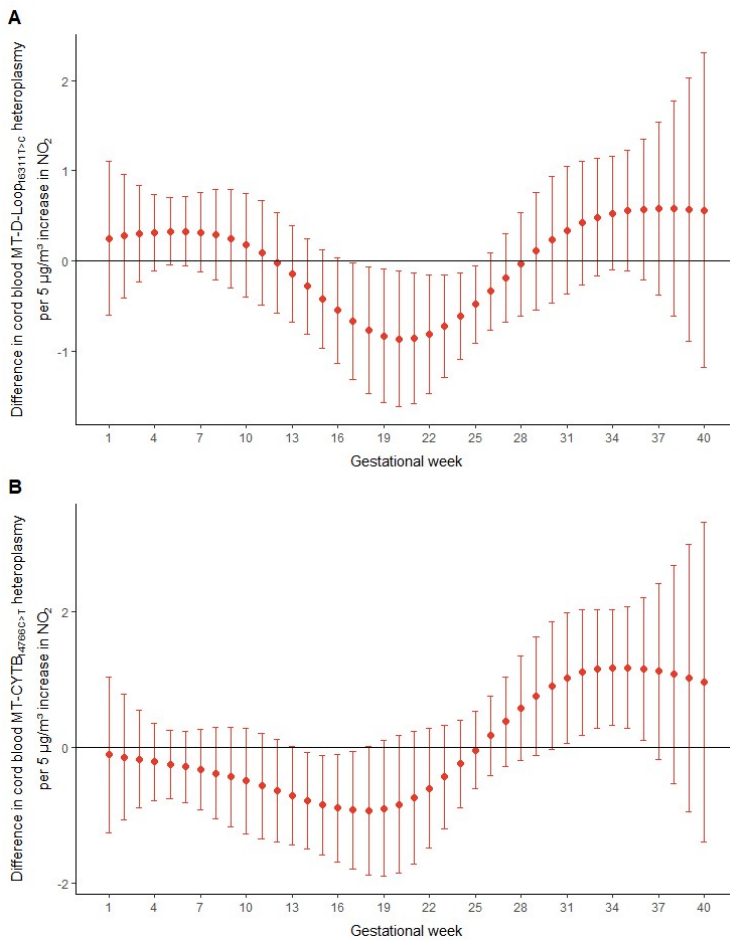
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236 week 32 – 36, with the largest effect in week 36 (1.35%; 95% CI: 0.22 to 2.49; p = 0.02; **Supplementary**
237 **Table S6**).

238



239
240 **Figure 2: Difference in cord blood mitochondrial heteroplasmy in association with week-specific prenatal**
241 **exposures to NO₂ for (A) MT-D-Loop_{16311T>C} and (B) MT-CYTB_{14766C>T} (n = 200). Week-specific estimates are given as**

242 % difference in VAF per 5 $\mu\text{g}/\text{m}^3$ increment in NO_2 . Models were adjusted for gestational age, newborn's sex, cord
243 blood mtDNA content, ethnicity, maternal age, socioeconomic status, pre-pregnancy BMI, smoking during
244 pregnancy, parity, and season of delivery. Error bars stand for the 95% CI for each weekly estimate.

245

246 DISCUSSION

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

255 Mitochondria are sensitive to damage induced by oxidative stress, which can be generated by various air
256 pollutants, including polycyclic aromatic hydrocarbons (PAHs), carbon monoxide (CO), volatile organic
257 compounds (VOCs), NO_2 , and PM (32). Both prenatal $\text{PM}_{2.5}$ (33) and NO_2 (16) exposure were linked with
258 lower mtDNA content, an indicator of mitochondrial (dys)function, in cord blood and placental tissue,
259 respectively. In contrast, little evidence is available linking prenatal air pollution to mtDNA mutations. *In*
260 *utero* exposure to $\text{PM}_{2.5}$ was linked with the cord blood MT-ND4L_{10550A>G} variant in newborns (34). In
261 addition, another study observed an association between placental mtDNA mutational load and prenatal
262 $\text{PM}_{2.5}$ exposure. These mutations were predominantly found in MT-ND5, MT-ATP6, and MT-ATP8 (13). The
263 association between *in utero* air pollution exposure and these mtDNA mutations were mainly observed in
264 mid- to late-pregnancy, which is consistent with our results. Our study showed a similar borderline trend
265 with mutational load in cord blood and prenatal NO_2 exposure in the second trimester of pregnancy.

266 We identified that MT-D-Loop_{16311T>C} heteroplasmy was associated with prenatal NO₂ exposure. The
267 mtDNA contains a hypervariable, non-coding displacement loop, also known as the D-Loop, which acts as
268 a promoter for both the heavy and light strands of the mtDNA and harbors most of the regulatory
269 sequences related to mtDNA replication, transcription, and translation (35-37). This D-Loop contains three
270 hypervariable regions: HV1 (positions 16024 – 16383), HV2 (positions 57 – 372), and HV3 (positions 438 –
271 574) (38). MT-D-Loop_{16311T>C} is a SNP in HV1, which was also the most common SNP identified in a sequence
272 analysis of hypervariable regions in ten unrelated Iranian families (39). Several studies linked mutations in
273 the mitochondrial D-Loop with cancer (37, 40, 41). As the D-Loop region regulates the replication,
274 transcription, and translation of mtDNA, mutations in this region might cause impaired mitochondrial
275 function due to the abnormal expression of mitochondrial proteins (35, 36, 42). Secondly, prenatal NO₂
276 exposure was linked with MT-CYTB_{14766C>T}. The *MT-CYTB* gene encodes one of the proteins of complex III,
277 which plays a role in oxidative phosphorylation. Mutations in this gene can cause mitochondrial complex
278 III deficiency, which is characterized by muscle weakness and pain. Individuals with higher heteroplasmy
279 levels of these mutations can also encounter problems with their liver, kidneys, heart, and brain (43). The
280 mutation MT-CYTB_{14766C>T} induces a codon change from ACT>ATT, causing a change in amino acid from
281 threonine to isoleucine, classifying it as a nonsynonymous SNP (44). One study reported that MT-
282 CYTB_{14766C>T} was significantly different in patients with normal-tension glaucoma, compared with controls
283 (45). ClinVar, a database of the relationships between human genetic variants and diseases (46), reported
284 a link between MT-CYTB_{14766C>T} and familial breast cancer (47) and Leigh syndrome (48). However, no
285 scientific reference was available for these conditions. Based on literature, none of the 28 included SNPs
286 were reported as highly pathogenic. Only one (i.e., rs267606617) out of ten pathogenic mtDNA variants
287 often found in mitochondrial disease (49) was identified once in our study population, which is linked with
288 maternally transmitted non-syndromic deafness (50).

289 More research concerning the identified SNPs in our study is warranted. Several studies reported
290 associations between other mitochondrial SNPs and health disorders, such as obesity (51), BMI (52), body
291 fat mass (53), blood pressure and fasting blood glucose levels (54), and MELAS (55). A mitochondrial
292 genome-wide association study (GWAS) identified ten SNPs that were associated with HDL cholesterol and
293 one SNP with triglycerides levels (56). Particularly individuals with the highest levels of heteroplasmy of
294 MT-TRNL1_{3243A>G} had a higher risk of all-cause, dementia, and stroke mortality (57). Also, in cancer
295 development, studies reported a heteroplasmy-shifting phenomenon as a potential biomarker of tumour
296 progression and treatment response (58). For example, changes in heteroplasmy levels were observed in
297 MT-COX1_{6419A}, which was heteroplasmic in normal samples while homoplasmic in tumour samples (59).

298 This is the first study to show that prenatal NO₂ exposure is associated with cord blood mitochondrial
299 mutations and suggests two critical windows of exposure in mid- to late-pregnancy. It has already been
300 reported that the placenta and mitochondria establish structural changes during the gestational period
301 (60). Initially in the first trimester, the embryo develops in a low oxygen environment, promoting
302 organogenesis, while in the third trimester, the rapid growth of the foetus demands higher oxygen levels
303 (61). Between gestational week 10 – 12, the maternal blood flow to the placenta is established (62) and
304 around the same time, mitochondrial respiration and placental mtDNA content increased (60). These
305 changes may indicate an increase in mitochondrial biogenesis, which in turn results in enhanced
306 antioxidant defences (60, 63). Mitochondrial biogenesis may be initiated as a response to adverse
307 conditions, such as preeclampsia or intrauterine growth restriction (IUGR), as these conditions were linked
308 with an increase in placental mtDNA content (64). A possible explanation of the observed negative
309 associations between prenatal NO₂ exposure and MT-D-Loop_{16311T>C} and MT-CYTB_{14766C>T} in the second
310 trimester of pregnancy might be an increase in mitochondrial biogenesis, resulting in improved antioxidant
311 defences. In contrast, MT-CYTB_{14766C>T} was positively associated with *in utero* exposure to NO₂ in the third
312 trimester, which can be explained by the fact that oxidative stress increased with gestational age (62).

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

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

326 SUPPORTING INFORMATION

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

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342 **AUTHOR CONTRIBUTIONS**

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

347 **COMPETING INTERESTS**

348 The authors declare no competing interests.

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