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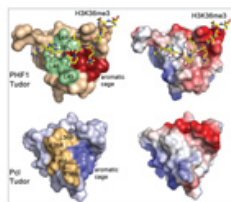
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**Key highlights**  
Methylation of tubulin binding sites in placental proteins is linked to the methylation of tubulin and other genes exposed to air pollution and stress in 2000.  
Placental methylation modification after early life exposure to environmental particulate matter is stable.



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### Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study

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# Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study

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**Keywords:** air pollution, DNA methylation, epigenetics, mitochondria, mitochondrial DNA content, mitochondrial DNA methylation, particulate matter, placenta

**Abbreviations:** 12S rRNA, Mitochondrial ribosomal 12S rRNA; *D-loop*, Displacement loop; IQR, Interquartile range; mtDNA, Mitochondrial DNA; mtDNMT1, Mitochondrial isoform of nuclear-encoded DNA methyltransferase enzyme 1; *MT-RNR1*, Mitochondrial region RNR1; PM, Particulate matter.

Most research to date has focused on epigenetic modifications in the nuclear genome, with little attention devoted to mitochondrial DNA (mtDNA). Placental mtDNA content has been shown to respond to environmental exposures that induce oxidative stress, including airborne particulate matter (PM). Damaged or non-functioning mitochondria are specifically degraded through mitophagy, exemplified by lower mtDNA content, and could be primed by epigenetic modifications in the mtDNA. We studied placental mtDNA methylation in the context of the early life exposome. We investigated placental tissue from 381 mother-newborn pairs that were enrolled in the ENVIRONAGE birth cohort. We determined mtDNA methylation by bisulfite-pyrossequencing in 2 regions, i.e., the *D-loop* control region and 12S rRNA (*MT-RNR1*), and measured mtDNA content by qPCR. PM<sub>2.5</sub> exposure was calculated for each participant's home address using a dispersion model. An interquartile range (IQR) increment in PM<sub>2.5</sub> exposure over the entire pregnancy was positively associated with mtDNA methylation (*MT-RNR1*: +0.91%,  $P = 0.01$  and *D-loop*: +0.21%,  $P = 0.05$ ) and inversely associated with mtDNA content (relative change of -15.60%,  $P = 0.001$ ) in placental tissue. mtDNA methylation was estimated to mediate 54% [ $P = 0.01$  (*MT-RNR1*)] and 27% [ $P = 0.06$  (*D-loop*)] of the inverse association between PM<sub>2.5</sub> exposure and mtDNA content. This study provides new insight into the mechanisms of altered mitochondrial function in the early life environment. Epigenetic modifications in the mitochondrial genome, especially in the *MT-RNR1* region, substantially mediate the association between PM<sub>2.5</sub> exposure during gestation and placental mtDNA content, which could reflect signs of mitophagy and mitochondrial death.

## Introduction

Prenatal life is an important window of susceptibility to adverse effects of environmental exposures. In epidemiological studies, it is well established that exposure to airborne particulate matter (PM) and tobacco smoke during gestation increases the risk of low birth weight and preterm birth.<sup>1–3</sup> While the specific mechanisms behind fetal programming are still poorly understood, a large body of evidence suggests the human placenta as a main target organ since it serves as the gatekeeper between

mother and fetus.<sup>4</sup> Indeed, the placenta is the first organ to be fully developed during pregnancy. It is in contact with all nutritional, hormonal, and other environmental stress factors throughout pregnancy. During its 9 months' lifespan, the human placenta records morphological, functional, genetic, and epigenetic information, representing thus a molecular "footprint" that can reveal the impact of *in utero* exposures on the fetus.<sup>5</sup>

The placenta is a unique vascular organ that requires a constant and abundant source of energy. Mitochondria are intracellular organelles providing cellular energy through the production

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of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. In humans, these powerhouses contain multiple copies of double stranded, circular mitochondrial DNA (mtDNA). Mitochondria, and associated mtDNA copies, may change in number or mass due to mitophagy, a specific form of autophagy in which dysfunctional or damaged mitochondria are removed and, thus, is critical for maintaining proper cellular function.<sup>6,7</sup> Analyzing mitochondrial mass by qPCR (i.e., mtDNA content) has been used as a quantitative method for specifically monitoring the last step of the degradation process of mitophagy in mammalian cells.<sup>8</sup> As such, alterations in mtDNA content are a marker of mitochondrial damage and dysfunction<sup>9</sup> and has been identified as an etiological determinant in a variety of human diseases, such as diabetes, obesity, cardiovascular disease, and cancer.<sup>10</sup> Changes in fetal mtDNA content may represent a biological effect along the path linking air pollution to effects on the fetus, such as birth weight. Previously, we showed a lower mtDNA content in placental tissue in association with *in utero* exposure to PM<sub>10</sub> (PM with aerodynamic diameter  $\leq 10 \mu\text{m}$ ),<sup>11</sup> which reflects signs of mitophagy and mitochondrial death.

Most research to date has focused on epigenetic modifications in the nuclear genome, with little attention devoted to mtDNA. Not up until the discovery of a mitochondrial isoform of nuclear-encoded DNA methyltransferase enzyme 1 (mtDNMT1) by Shock et al.,<sup>12</sup> there was controversy and inconclusive evidence of DNA methylation in the mitochondrial genome. Although mtDNA methylation, by means of 5-methylcytosine and 5-hydroxymethylcytosine, has been increasingly well established after this breakthrough and led to several significant disease-related studies,<sup>13,14</sup> such as cervical<sup>15</sup> and colorectal cancer,<sup>16</sup> non-alcoholic fatty liver disease,<sup>17</sup> and aging,<sup>18</sup> there are still conflicting reports in the literature regarding the existence and function of mtDNA methylation.<sup>19,20</sup> Indeed, the functional consequences of this epigenetic mark are still under investigation but emerging evidence suggests a role in transcription modification of the mitochondrial genome.<sup>16,17</sup> For example, Bellizzi et al.<sup>21</sup> confirmed that mtDNA is methylated at the displacement loop (*D-loop*), a crucial promoter region from which mtDNA replication and transcription are initiated. Furthermore, some authors have suggested that aberrant DNA methylation in the mitochondrial *RNRI* (*MT-RNRI*) sequence, encoding for the mitochondrial ribosomal 12S rRNA (12S rRNA), could affect normal function and integrity of the mitochondrial ribosome.<sup>22</sup> Structural changes of 12S rRNA lead to instability of the small subunit of the ribosome and abolishes translation of mtDNA-encoded RNAs into proteins.<sup>23</sup> Therefore, it is plausible that epigenetic modifications at important hotspots in the mtDNA can regulate replication and/or transcription of the mtDNA.

Whether exposure to PM during gestation influences methylation patterns of the mitochondrial genome in placental tissue is not known. In the framework of the ENVIRONAGE birth cohort (ENVIRONmental influence ON early AGEing), we hypothesized that, in addition to mtDNA content, methylation at the *D-loop* and *MT-RNRI* region (Fig. 1) in placental tissue is associated with airborne PM<sub>2.5</sub> exposure during gestation. To

elucidate possible mechanisms that regulate mitochondrial function, we also examined mtDNA methylation as potential mediator between early life exposure to airborne PM and placental mtDNA content.

## Results

### Participant's characteristics and exposure levels

Demographic and pregnancy-related characteristics of the 381 mother-newborn pairs are reported in Table 1. Briefly, mean maternal age was 29.0 y (range: 18–42 y) and more than 50% of the mothers were highly educated. Pre-gestational body mass index (BMI) of the participating mothers averaged 24.3 (SD = 4.5) kg/m<sup>2</sup>. Most women (66.9%; n = 255) never smoked cigarettes and 65 women (17.1%) stopped smoking before pregnancy (median with 25th – 75th percentile: 1.5 (0–5) years), whereas 61 women (16.0%) reported to have smoked during pregnancy (on average 7.8 cigarettes per day). The newborns, 193 of which were girls (50.7%), had a mean gestational age of 39.2 weeks (range: 35–42) and comprised 200 (52.5%) primiparous and 141 (37.0%) secundiparous. About 90% (n = 332) of the newborns were Europeans of Caucasian ethnicity. Birth weight averaged 3431 (425) grams. Nineteen (5.0%) newborns were delivered with a caesarian section.

Table 2 displays the daily outdoor PM<sub>2.5</sub> exposure levels averaged for the entire pregnancy and each of the 3 trimesters of pregnancy. Average (interquartile range (IQR): 25th–75th percentile) trimester-specific PM<sub>2.5</sub> exposure was 16.0 (11.8–19.6)  $\mu\text{g}/\text{m}^3$  for the first trimester, 16.9 (12.2–20.4)  $\mu\text{g}/\text{m}^3$  for the second trimester, 17.3 (11.9–21.7)  $\mu\text{g}/\text{m}^3$  for the third trimester, and 16.7 (15.2–18.2)  $\mu\text{g}/\text{m}^3$  for the entire pregnancy.

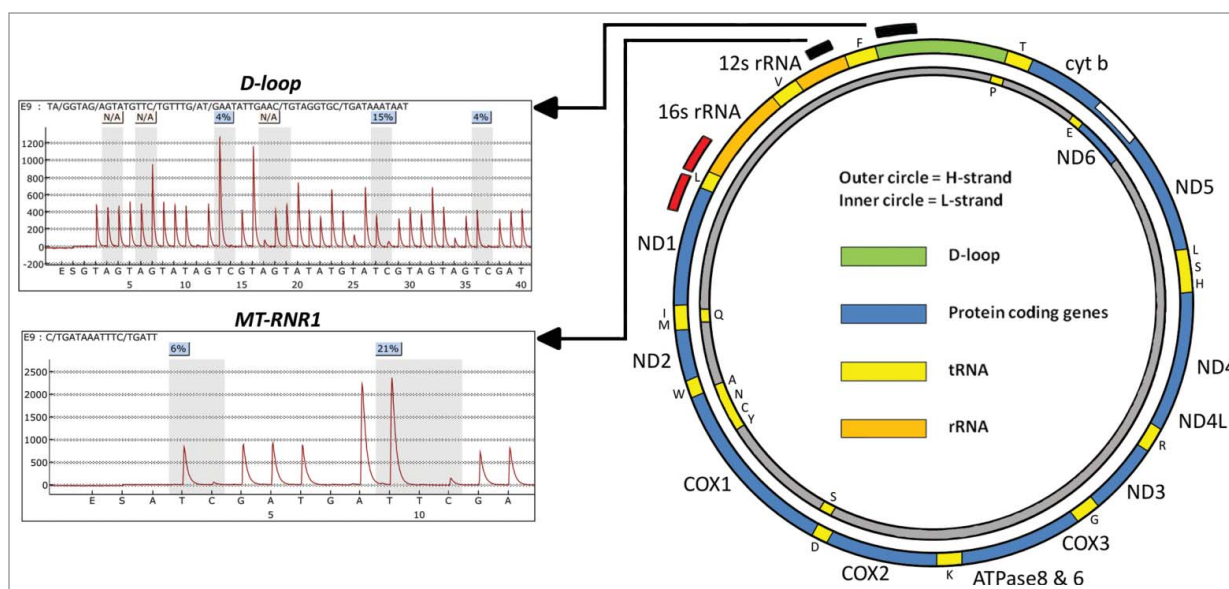
### Placental mtDNA methylation and mtDNA content in association with PM<sub>2.5</sub> exposure during gestation

We found a positive association of placental mtDNA methylation (*MT-RNRI* and *D-loop*, separately as well as combined) with PM<sub>2.5</sub> exposure during the entire gestation, which was independent of gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception (Table 3). The association was most pronounced in the first trimester of pregnancy (*MT-RNRI*: 1.27%, 95% CI: 0.23 to 2.32%; *D-loop*: 0.44%, 95% CI: 0.12 to 0.75%; n = 356 for an IQR increment of PM<sub>2.5</sub> levels).

Furthermore, when adjusting for the aforementioned covariates, a lower placental mtDNA content of –15.60% (95% CI: –23.92 to –6.38%) was observed for an IQR increment of PM<sub>2.5</sub> exposure over the entire pregnancy, and the association was most pronounced in the third trimester (–23.58%, 95% CI: –36.27 to –8.37%) (Table 3).

### Correlation of placental mtDNA content and mtDNA methylation

To explore the functional significance of the association of mtDNA methylation with exposure to PM<sub>2.5</sub>, we evaluated the correlation between placental mtDNA content, a measure of



**Figure 1.** Location of mitochondrial targets in the human mitochondrial genome. The double stranded, circular mtDNA (16569 bp) contains 37 genes, specifying 13 polypeptides (blue boxes), 2 rRNAs, i.e., 12S rRNA and 16S rRNA (orange boxes), and 22 tRNAs (yellow boxes). The single letter codon associated with the yellow box refers to the amino acid. The *D-loop* region (green box) contains the origin of replication and is the place where promoters initiate transcription of the H-strand. The target location for DNA methylation analysis in the *D-loop* and *MT-RNR1* region are denoted by the black arcs, with magnified pyrograms. The red arcs depict the targets for mtDNA content analysis (*MTF3212/R3319* and *ND1*). NADH dehydrogenase (ND); Cytochrome c oxidase (COX); F1F0-ATP synthase (ATPase); Cytochrome b (Cyt b).

damaged mitochondria, and mtDNA methylation. Newborns with lower mtDNA content in placental tissue exhibited higher levels of methylation in both mitochondrial regions as shown in **Figure 2**, and remained significant after adjustment for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception (*MT-RNR1*:  $\beta = -0.04 \pm 0.002$ ,  $P < 0.0001$ ; *D-loop*:  $\beta = -0.10 \pm 0.01$ ,  $P < 0.0001$ ;  $n = 356$ ).

#### Mediating effects of mtDNA methylation on the association of PM<sub>2.5</sub> exposure and mtDNA content

We used mediation analysis to test if the negative association between placental mtDNA content and PM<sub>2.5</sub> exposure during gestation was mediated by increased mtDNA methylation in both mitochondrial regions. All assumptions for mediation analysis were satisfied and we used average methylation levels of *MT-RNR1* and *D-loop* separately. We considered the exposure averaged over the entire pregnancy because of the discrepancy in trimester effect of PM<sub>2.5</sub> exposure on mtDNA methylation (first trimester) and mtDNA content (third trimester). Estimates of the proportion of mediation indicated that placental *MT-RNR1* and *D-loop* methylation mediated respectively 54% (95% CI: 31 to 60%,  $P = 0.01$ ) and 27% (95% CI: -3 to 36%,  $P = 0.06$ ) of the inverse association between PM<sub>2.5</sub> exposure and placental mtDNA content (**Fig. 3**).

#### Sensitivity analysis

In a sensitivity analysis, we repeated the main statistical analysis after additional adjustment of caesarian section but this did not alter the reported association between PM<sub>2.5</sub> exposure and mtDNA content or mtDNA methylation. In addition, we

repeated all statistical analyses while excluding preterm born infants (< 37 weeks,  $n = 13$ ) and also excluding women who smoked during pregnancy ( $n = 61$ ). This did not alter the reported associations between mtDNA content or mtDNA methylation and PM<sub>2.5</sub> exposures (**Table S1**).

The associations in the main analysis between the first trimester PM<sub>2.5</sub> exposure with the *D-loop* and *MT-RNR1* region differed by CpG position in magnitude and significance. For example, methylation levels at position 2 and 3 of the *D-loop* region were strongest associated with the first trimester PM<sub>2.5</sub> exposure whereas the CpG positions of the *MT-RNR1* region differed in magnitude and significance (**Table S2**).

Neither methylation levels of the *MT-RNR1* nor the *D-loop* region were associated with low birth weight (*MT-RNR1*:  $P = 0.58$  and *D-loop*:  $P = 0.57$ ) or small for gestational age (*MT-RNR1*:  $P = 0.66$  and *D-loop*:  $P = 0.72$ ) (**Table S3**).

## Discussion

The key finding of our study is that placental mtDNA methylation is positively associated with PM<sub>2.5</sub> exposure during gestation, especially in the first trimester of pregnancy. Further, these epigenetic modifications in the mitochondria substantially mediate the association between PM<sub>2.5</sub> exposure during gestation and placental mtDNA content, demonstrating an intermediate mechanism of mtDNA alterations that could reflect mitophagy and mitochondrial death. To date, epigenetic mitochondrial modifications in the context of exposures in the early life environment have not been reported.

**Table 1.** Characteristics of mother-newborn pairs (n = 381)

Characteristics	Mean	± SD or range and number (%)
<b>Maternal</b>		
Age, y	29.0	(18–42)
Pre-gestational BMI, kg/m <sup>2</sup>	24.3	± 4.5
Maternal education		
Low	47	(12.7%)
Middle	132	(34.7%)
High	200	(52.6%)
Smoking		
Never-smoker	255	(66.9%)
Past-smoker	65	(17.1%)
Smoker	61	(16.0%)
Parity		
1	200	(52.5%)
2	141	(37.0%)
≥ 3	40	(10.5%)
Mode of delivery		
Caesarian section	19	(5.0%)
<b>Newborn</b>		
Gender		
Female	193	(50.7%)
Ethnicity		
European	332	(87.1%)
Non-European	49	(12.9%)
Gestational age, w	39.2	(35–42)
Season at conception		
Winter	90	(23.6%)
Spring	87	(22.8%)
Summer	118	(31.0%)
Fall	86	(22.6%)
Apgar score after 5 min		
≤ 8	22	(5.7%)
9	110	(28.9%)
10	249	(65.4%)
Birth weight, g	3431	± 425
<i>MT-RNR1</i> mtDNA methylation, %*	9.54	± 4.2
<i>D-loop</i> mtDNA methylation, % <sup>‡</sup>	3.60	± 1.28 (n = 356)
mtDNA content <sup>#</sup>	1.04	(0.45–2.45)

\*Measured in placental tissue.

<sup>#</sup>Geometric mean with 10th–90th percentiles.

Epigenetic changes, of which DNA methylation is the most commonly characterized, can occur throughout the course of life, but much of the epigenome is already established in germ cells and embryos as it appears to be particularly important for the regulation of embryonic growth and placental development.<sup>25</sup> Up until recently, it was thought that epigenetic modifications could only occur in nuclear DNA and not in mtDNA. Despite the discovery of the mtDNMT1 enzyme by Shock et al.<sup>12</sup> and studies demonstrating mtDNA methylation in humans,<sup>21,22,26</sup>

**Table 2.** Exposure characteristics (n = 381)

Exposure variable	Mean	± SD	25th percentile	75th percentile
<b>PM<sub>2.5</sub>, µg/m<sup>3</sup></b>				
1st trimester	16.0	± 5.3	11.8	19.6
2nd trimester	16.9	± 5.0	12.2	20.4
3rd trimester	17.3	± 5.8	11.9	21.7
Entire pregnancy	16.7	± 2.3	15.2	18.2

mammals,<sup>21,23</sup> and cultured cells,<sup>21,27</sup> there are still controversial studies reporting complete absence of mtDNA methylation in human cells lines<sup>19</sup> or questioning the clinical usefulness of this biological phenomenon.<sup>20</sup> Nevertheless, the rationale of studying mtDNA methylation is underscored by its role in the etiology of a variety of human diseases<sup>13,15–17</sup> and aging.<sup>18</sup> Growing evidence suggests mitochondrial epigenetics as a novel mechanism to understand the pathobiology of diseases with a mitochondrial dysfunction involvement.<sup>14,17</sup> An important hotspot for epigenetic regulation in the mitochondrial genome is the displacement loop or *D-loop*.<sup>21</sup> DNA methylation levels in this control region are suggested to play an important role in modulating either replication or transcription of mtDNA since nearly the entire mitochondrial genome transcribes from this region.<sup>28</sup> In tumor and corresponding noncancerous tissues from colorectal cancer patients, Feng et al.<sup>16</sup> observed a correlation between *D-loop* methylation and expression of *ND2*, a subunit of NADH encoded by mtDNA, which is a rate-limiting enzyme of the oxidative phosphorylation system. In addition, the methylation status of *ND6*, which is another crucial subunit for complex I assembly, significantly impacts the transcriptional regulation of the gene,<sup>17</sup> an effect probably dependent on the enhanced expression of *DNMT1*.<sup>12</sup> Our other studied gene *MT-RNR1* encodes for the 12S rRNA protein, which is critical for normal function and integrity of the mitochondrial ribosome. Methylation of *MT-RNR1* may cause malfunction of mitochondrial ribosomes and abolished translation of mtDNA-encoded RNAs into proteins.<sup>23</sup> A preliminary study in human blood showed that the methylation status of mitochondrial *MT-RNR1* gene decreased during aging.<sup>29</sup>

Epigenetic modifications represent a potential link between adverse insults and altered fetal development. Previously, we have shown that PM<sub>2.5</sub> exposure during pregnancy affects global DNA methylation levels in placental tissue,<sup>30</sup> while in this study, we investigated the impact of PM<sub>2.5</sub> exposure upon epigenetics of placental mitochondria. Using the bisulfite-pyrosequencing approach, we were able to detect subtle differences in methylation levels of specific regions in the mitochondrial genome (Fig. 1). Our observations in newborns are partially in accordance with steel workers. Byun and colleagues<sup>22</sup> found a significant positive association between metal-rich PM<sub>1</sub> and DNA methylation at the *MT-RNR1* sequence, but not at the *D-loop*, in blood leukocytes of steel workers, whereas we found positive associations of PM<sub>2.5</sub> exposure with methylation at both mitochondrial regions in placental tissue. It is noteworthy to mention that we observed an inverse correlation between placental mtDNA methylation and mtDNA content in contrast to a positive correlation observed in blood leukocytes.<sup>22</sup> Previous investigations have shown that mtDNA content is variable and fluctuates during aging,<sup>31,32</sup> under the influence of different environmental factors,<sup>33</sup> and the tissue investigated,<sup>11,31,34</sup> as well as different effects from recent versus long-term exposures. Nevertheless, we found similar results when we compared placental mtDNA content between mothers who continued smoking vs. non-smoking pregnant mothers (i.e., lower mtDNA content in smokers; relative difference of –21.98%, 95% CI: –41.10 to

**Table 3.** PM<sub>2.5</sub> exposure during the different periods of pregnancy in association with placental mtDNA methylation and placental mtDNA content

Variable	First trimester		Second trimester		Third trimester		Entire pregnancy	
	β	(95% CI)	β	(95% CI)	β	(95% CI)	β	(95% CI)
mtDNA methylation*								
<i>MT-RNR1</i> , %	1.27	(0.23 to 2.32) <sup>†</sup>	0.19	(−0.80 to 1.16)	1.04	(−0.20 to 1.86)	0.91	(0.56 to 4.18) <sup>†</sup>
<i>D-loop</i> , % <sup>#</sup>	0.44	(0.12 to 0.75) <sup>†</sup>	0.09	(−0.22 to 0.39)	0.04	(−0.29 to 0.36)	0.21	(−0.003 to 1.02)
Combined, %	0.75	(0.16 to 1.34) <sup>†</sup>	0.10	(−0.47 to 0.65)	0.46	(−0.23 to 0.96)	0.47	(0.20 to 2.23) <sup>†</sup>
mtDNA content, %**	−7.57	(−20.78 to 7.86)	−15.19	(−28.34 to 0.38)	−23.58	(−36.27 to −8.37) <sup>†</sup>	−15.60	(−23.92 to −6.38) <sup>†</sup>

\*β represents an absolute change in percentage methylation for an IQR increment of PM<sub>2.5</sub> exposure (μg/m<sup>3</sup>).

\*\*β represents a relative change in placental mtDNA content for an IQR increment of PM<sub>2.5</sub> exposure (μg/m<sup>3</sup>).

<sup>#</sup>Data available for 356 individuals.

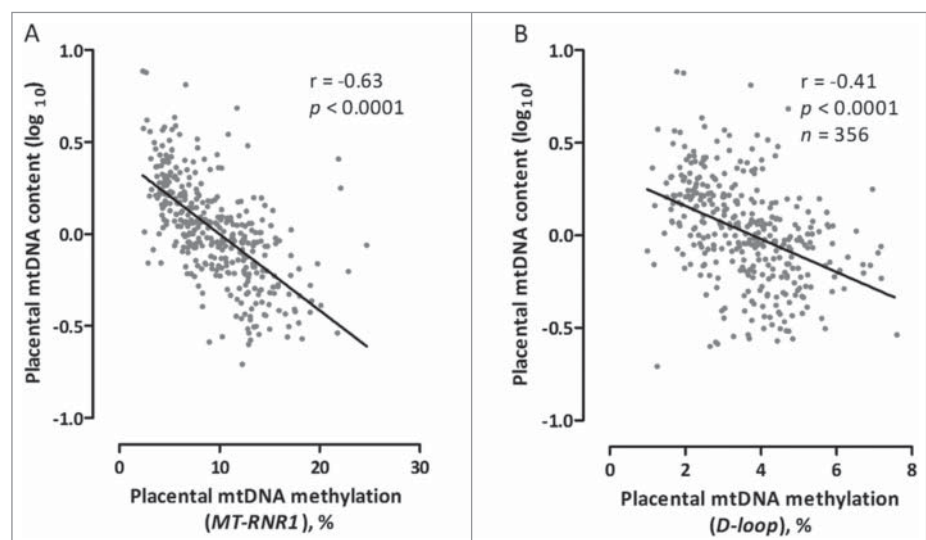
<sup>†</sup>P-value significantly lower than 0.05. All models are adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

−0.86%,  $P = 0.01$ ). Considering smoking as a personal form of intensive exposure to particulate air pollution, this consistency with ambient air pollution adds confidence to the causal interpretation of our association between PM<sub>2.5</sub> exposure and placental mtDNA content. In addition, a study that employed side-by-side epigenome-wide methylation and gene expression arrays in placentas of smokers, showed a significant correlation between methylation and gene expression of 438 genes that were involved in mitochondrial dysfunction, oxidative phosphorylation, and hypoxia pathways.<sup>5</sup>

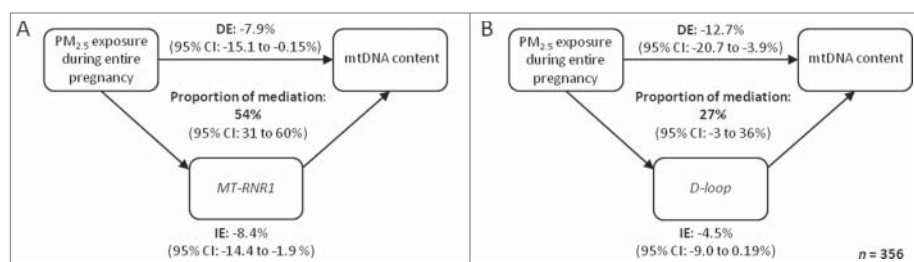
The mitochondrion is an important environmental biosensor and the findings of our study brings us one step closer to unravel the relevance of mtDNA content and mtDNA methylation to exposure-related human diseases.<sup>14,35</sup> Our data indicate that mtDNA methylation, especially at the *MT-RNR1* region, might be a method by which mitochondrial biogenesis and function is regulated. We observed that the association between PM<sub>2.5</sub> exposure and mtDNA methylation is most pronounced in the first trimester and the association between PM<sub>2.5</sub> exposure and mtDNA content most pronounced in the third trimester of pregnancy. Given the functional relevance of the *D-loop* region and the 12S rRNA, encoded by *MT-RNR1*, in controlling replication, transcription, and translation of the mtDNA, we postulate that aberrant mtDNA methylation at these hotspots could interfere with mtDNA biogenesis. This premise holds true when we consider the results of our mediation analysis indicating that a substantial proportion of the association between PM<sub>2.5</sub> exposure and placental mtDNA content was mediated by mtDNA methylation and, therefore, strengthens the evidence of an intermediate mechanism of mtDNA alterations. Our findings of lower mtDNA content in placental tissue in association with prenatal PM exposure might reflect increased mitophagy and

mitochondrial death, or it could indicate a decline in mitochondrial biogenesis due to demethylation of the mtDNA-specific polymerase gamma A (*POLGA*)<sup>36</sup> or a reduction in levels of *PPARGCIA* (peroxisome proliferator-activated receptor-coactivator1α), the master regulator of mitochondrial biogenesis.<sup>37</sup> Furthermore, depletion of mtDNA results in significant changes in the methylation pattern of a number of nuclear genes,<sup>38</sup> indicating a possible effect of mitochondrial dysfunction on the epigenetic landscape of the nuclear genome.<sup>39</sup>

We acknowledge several limitations in the present study. Although we reported effects on 2 mtDNA regions separately and performed a combined analysis of these 2 regions, we cannot extrapolate these findings to the entire mitochondrial genome. We selected 2 regions with potential functional impacts, including a promoter region and a key rRNA sequence (Fig. 1). Future work is warranted to determine whether DNA methylation and hydroxymethylation in other regions of the mtDNA, such as *ND2* and *ND6*, which have a biological significance for



**Figure 2.** Correlation of mtDNA methylation and mtDNA content in placental tissue. Placental mtDNA content is log<sub>10</sub>-transformed. Methylation levels are indicated as absolute percentage levels. (A) *MT-RNR1* methylation levels. (B) *D-loop* methylation levels.



**Figure 3.** Estimated proportion of effects of  $PM_{2.5}$  exposure during gestation on mtDNA content mediated through changes in mtDNA methylation. The figure displays placental mtDNA methylation as mediator [(A) *MT-RNR1*; (B) *D-loop*], the estimates of indirect effect (IE), the estimates of the direct effect (DE), and proportion of mediation (IE/DE+IE). The effects are relative changes in placental mtDNA content for an IQR increment of  $PM_{2.5}$  exposure. The mediation model was adjusted for gender, age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.

mitochondrial function, are specifically sensitive to air pollutants, and whether these changes are linked to functional alterations in expression of mitochondrial genes. Secondly, we postulate that aberrant mtDNA methylation could interfere with mtDNA biogenesis. However, because our measurements were performed at birth, we cannot ascertain the temporal sequence, although our proposed biological model seems plausible. Indeed, in epidemiological settings, even by use of mediation analysis, the biological direction of observed associations cannot be determined. Lastly, we can only speculate about the potential health consequences of altered mtDNA methylation and mtDNA content in placental tissue in response to  $PM_{2.5}$  exposure and, therefore, a follow-up study is warranted. Of note, a preliminary study showed differential mtDNA methylation in growth restricted placentas and underscores a possible functional relevance of mtDNA methylation in the placenta.<sup>40</sup> In our study, we did not find an association between altered placental mtDNA methylation and adverse pregnancy outcomes such as low birth weight or small for gestational age.

In summary, our study indicates mtDNA methylation as an early molecular event involved, at least partially, with a decrease in placental mtDNA content, which might reflect mitophagy, in association with prenatal exposure to  $PM_{2.5}$ . Although concise pathological roles of mitochondrial methylation during development need to be further elucidated, our findings open a new area to the molecular epidemiological understanding of mitochondrial alterations in early life.

## Materials and Methods

### Study population

We recruited 400 mother-newborn pairs (only singletons) from the ongoing ENVIRONAGE birth cohort as previously described.<sup>11</sup> The study participants lived in the Flemish region of Belgium. The placenta could not be collected from 10 newborns, DNA yield was insufficient for 4 placentas and 5 newborns had missing data for PM exposure (lived outside Belgium). Therefore, the final sample included 381 newborns. Written informed consent was obtained from all participating mothers when they

arrived at the hospital for delivery and was in accordance with procedures approved by the Ethical Committee of Hasselt University and East-Limburg Hospital in Genk. The study was conducted according to the principles outlined in the Helsinki Declaration for investigation of human subjects. The participation rate of eligible mothers (able to fill out a Dutch language questionnaire) was 56% and enrolment was spread equally over all seasons of the year. Study questionnaires were completed in the postnatal ward after delivery and provided detailed information on age, place of residence, maternal edu-

cation, occupation, pre-gestational BMI, smoking status, use of medication, alcohol consumption, parity, mode of delivery (vaginal or caesarian section), and newborn's ethnicity. Past-smokers were defined as those who had quit before pregnancy and smokers as having smoked before and during pregnancy. Maternal education was coded as low (no diploma or primary school), middle (high school) or high (college or university degree). Perinatal parameters such as birth date, gestational age, newborn's gender, birth weight and length, Apgar score, and ultrasonographic data were collected after birth.

### Exposure measurement

For each mother's residential address, we interpolated the regional background levels of  $PM_{2.5}$  (micrograms per cubic meter) using a spatial temporal interpolation method (Kriging)<sup>41</sup> that uses pollution data collected in the official fixed site monitoring network ( $n = 34$ ) and land cover data obtained from satellite images (Corine land cover data set) in combination with a dispersion model (dispersion modeling described by Lefebvre et al.<sup>42,43</sup>). This model chain provides daily  $PM_{2.5}$  values using data from the Belgian telemetric air quality network, point sources and line sources which are then interpolated to a high-resolution receptor grid. In the Flemish region of Belgium, the interpolation tool explained more than 80% of the temporal and spatial variability ( $R^2$ ).<sup>44</sup> The exposure during the entire pregnancy was calculated as the mean of all pregnancy days. The date of conception was estimated based on ultrasound data. We explored potential critical windows of exposures during pregnancy using daily mean  $PM_{2.5}$  concentrations averaged over various periods. The exposure windows of interest included each of the 3 trimesters of pregnancy, with trimesters being defined as: 1–13 weeks (first trimester), 14–26 weeks (second trimester), and 27 weeks to delivery (third trimester). Address changes during the period of pregnancy were taken into account when calculating the exposure windows ( $n = 38$ ; 9.9%).

### Placental sampling

Placentas were obtained in the delivery room and deep-frozen within 10 minutes. As described previously,<sup>30</sup> we took villous tissue (1 to 2  $cm^3$ ) at a fixed location from the fetal side of the

placenta, approximately 1–1.5 cm below the chorio-amniotic membrane, and preserved the biopsies at  $-80^{\circ}\text{C}$ . Chorio-amniotic membrane contamination was avoided by careful visual examination and dissection.

#### DNA methylation analysis

Genomic DNA was isolated from placental tissue ( $n = 381$ ) using the QIAamp DNA mini kit (Qiagen, Inc., Venlo, the Netherlands). We performed DNA methylation analysis by highly quantitative bisulfite-PCR pyrosequencing as previously described in detail.<sup>24</sup> Briefly, bisulfite conversions were performed using 1  $\mu\text{g}$  of extracted genomic DNA with the EZ-96 DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. We interrogated CpG sites within specific regions of the mitochondrial genome (*MT-RNR1* and *D-loop*) as described by Byun et al.<sup>22</sup> and Janssen et al.<sup>24</sup> (Fig. 1). Detailed information regarding primer sequences is given in Table S4. PCR amplification of regions of interest prior to pyrosequencing was performed in a total reaction volume of 30  $\mu\text{l}$ , using 15  $\mu\text{l}$  GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 10 pmol forward primer, 10 pmol reverse primer, 1  $\mu\text{l}$  bisulfite-treated genomic DNA and water. PCR products were purified and sequenced by pyrosequencing using the PyroMark Q96 MD Pyrosequencing System (Qiagen, Inc., Germantown, MD, USA). The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Samples were run in duplicate on 2 different plates from which the average methylation levels were used. The coefficient of variation for *MT-RNR1* was 3.7% and for *D-loop* 5.8%. The efficiency of the bisulfite-conversion process was assessed using non-CpG cytosine residues within the sequence. The between- and within-placenta variability, exemplified by the intra-class correlation coefficient, was evaluated in a subset of 19 placentas and was 58% vs. 42% ( $P = 0.009$ ) for *MT-RNR1* and 61% vs. 39% ( $P = 0.01$ ) for the *D-loop* region.<sup>24</sup>

#### mtDNA content analysis

mtDNA content was measured in placental tissue ( $n = 381$ ) by determining the ratio of 2 mitochondrial gene copy numbers (*MTF3212/R3319* and *ND1*) (Fig. 1) to 2 single-copy nuclear control genes (*RPLP0* and *ACTB*) using a quantitative real-time polymerase chain reaction (qPCR) assay as previously described<sup>11</sup> but with small modification. Briefly, 2.5  $\mu\text{l}$  diluted genomic DNA (5 ng/ $\mu\text{l}$ ) was added to 7.5  $\mu\text{l}$  mastermix consisting of Fast SYBR<sup>®</sup> Green I dye 2  $\times$  (5  $\mu\text{l}$ /reaction), forward and reverse primer (each 0.3  $\mu\text{l}$ /reaction), and RNase free water (1.9  $\mu\text{l}$ /reaction) to a final volume of 10  $\mu\text{l}$  per reaction. Primers (Table S5) were diluted to a final concentration of 300 nM in the master mix. Samples were run in triplicate in 384-well format. Real-time PCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with following thermal cycling profile: 20 s at  $95^{\circ}\text{C}$  (activation), followed by 40 cycles of 1 s at  $95^{\circ}\text{C}$  (denaturation), and 20 s at  $60^{\circ}\text{C}$  (annealing/extension), ending with melting curve analysis (15 s at  $95^{\circ}\text{C}$ , 15 s at  $60^{\circ}\text{C}$ , 15 s at  $95^{\circ}\text{C}$ ). qBase software (Biogazelle, Zwijnaarde, BE)

automatically averages triplicate measurements that pass quality control and normalizes the data to nuclear reference genes while correcting for run-to-run differences.<sup>45</sup>

#### Statistical analysis

For database management and statistical analysis, we used the SAS software program (version 9.2; SAS Institute Inc., Cary, NC, USA). mtDNA content was  $\log_{10}$ -transformed to improve normality. We measured methylation levels of 2 and 3 CpG sites for the *MT-RNR1* and *D-loop* region respectively (Fig. 1). Pearson correlation coefficients were used to assess correlations between adjacent CpG sites within one region (*MT-RNR1* or *D-loop*) and to assess the correlation between mtDNA content and mtDNA methylation. Within each region, the associations with individual CpG positions were similar in size and direction ( $r \geq 0.90$ , Fig. S1) and we assumed equal correlation between CpG positions in our models. We used 2 statistical modeling approaches to interpret mtDNA methylation data: 1) with mixed-effect models, we took into account each CpG dinucleotide position of both regions (combination of *MT-RNR1* and *D-loop*) to obtain a robust estimate of the average effect on DNA methylation in the mitochondrial genome; 2) with mixed-effect models, we took into account each CpG dinucleotide position of either *MT-RNR1* or *D-loop* to obtain an estimate of each region separately. We performed multiple linear regression to determine the effect-size of  $\text{PM}_{2.5}$  exposure during pregnancy on placental mtDNA content. All adjusted models were controlled for *a priori* chosen variables including gender, maternal age, gestational age, smoking, maternal education, parity, ethnicity, and season at conception.  $\beta$  regression coefficients represent a relative (mtDNA content) or absolute (mtDNA methylation) percentage change for an increment between the IQR (25<sup>th</sup>-75<sup>th</sup> percentile) in the independent variable. The Shapiro-Wilk statistic and Q-Q plots of the residuals were used to test the assumptions of all linear models.

We used mediation analysis to investigate potential mechanisms that underlie the association between the exposure variable ( $\text{PM}_{2.5}$ ) and outcome variable (mtDNA content) by examining how they relate to a third intermediate variable, the mediator (mtDNA methylation).<sup>46</sup> We accomplished this by decomposing the total effect into direct effects (exposure effect on outcome at a fixed level of the mediator) and indirect effects (exposure effect on outcome that operate through mediator levels). Mediation analysis usually requires a significant relation of the outcome to the exposure, a significant relation of the outcome to the mediator and a significant relation of the mediator to the exposure. This is based on the *a priori* assumption that a mediated effect is biologically plausible. For the mediation analysis, we considered  $\text{PM}_{2.5}$  exposure during the entire pregnancy and average methylation levels of each mitochondrial region. We used SAS macros provided by Valeri et al.<sup>46</sup>

In a sensitivity analysis, we assessed the association between  $\text{PM}_{2.5}$  exposure during pregnancy and mtDNA methylation or mtDNA content while excluding preterm births ( $< 37$  weeks) or women who smoked during pregnancy. In addition, we



examined whether the PM<sub>2.5</sub> exposure associations we observed in the main analysis were specific to certain CpG positions within the 2 mitochondrial regions. Finally, we investigated whether mtDNA methylation was associated with adverse pregnancy outcomes such as low birth weight using linear regression models and small for gestational age using logistic regression models.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Material

Supplemental data for this article can be found on the publisher's website.

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