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Prenatal ambient temperature exposure and cord blood and placental mitochondrial DNA content: Insights from the ENVIRONAGE birth cohort study

Eleni Renaers [a,1], Congrong Wang [a,1], Esmée M. Bijnens [a,b], Michelle Plusquin [a], Tim S. Nawrot [a,c], Dries S. Martens [a,*]

[a] Centre for Environmental Sciences Hasselt University Hasselt Belgium
[b] Department of Environmental Sciences Open University Heerlen Netherlands
[c] Department of Public Health and Primary Care Leuven University Leuven Belgium

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ABSTRACT

Background: Mitochondrial DNA content (mtDNAc) at birth is a sensitive biomarker to environmental exposures that may play an important role in later life health. We investigated sensitive time windows for the association between prenatal ambient temperature exposure and newborn mtDNAc.

Methods: In the ENVIRONAGE birth cohort (Belgium), we measured cord blood and placental mtDNAc in 911 participants using a quantitative real-time polymerase chain reaction. We associated newborn mtDNAc with average weekly mean temperature during pregnancy using distributed lag nonlinear models (DLNMs). Double-threshold DLNMs were used to study the relationships between ambient temperature and mtDNAc below predefined low (5th, 10th, 15th percentile of the temperature distribution) and above predefined high temperature thresholds (95th, 90th, 85th percentile of the temperature distribution).

Findings: Prenatal temperature exposure above the used high temperature thresholds was linked to lower cord blood mtDNAc, with the strongest effect in trimester 2 (cumulative estimates ranging from −21.4% to −25.6%). Placental mtDNAc showed positive and negative associations for high temperature exposure depending on the applied high temperature threshold. Negative associations were observed during trimester 1 using the 90th and 95th percentile threshold (−26.1% and –33.2% lower mtDNAc respectively), and a positive association in trimester 3 when applying the most stringent 95th percentile threshold (127.0%). Low temperature exposure was associated with higher mtDNAc for both cord blood and placenta. Cord blood mtDNAc showed a positive association in trimester 2 when using the 10th percentile threshold (11.3%), while placental mtDNAc showed positive associations during the whole gestation and for all applied thresholds (estimates ranging from 80.8% – 320.6%).

Interpretation: Our study shows that *in utero* temperature exposure is associated with differences in newborn mtDNAc at birth, with stronger associations observed in the placenta. These findings highlight the impact of prenatal ambient temperature exposure on mtDNAc during pregnancy.

1. Introduction

As global warming continues, exposure to extreme ambient temperatures becomes more frequent, mainly affecting vulnerable groups, including pregnant women and fetuses (Ebi et al., 2021; Xu et al., 2012). Exposure to extreme temperatures during pregnancy can disrupt maternal homeostasis, causing oxidative stress and inflammation (Cox et al., 2016; Martens et al., 2019; Kuehn and McCormick, 2017; Nyadanu et al., 2024). Studies have already linked prenatal ambient temperature exposure to adverse effects on birth outcomes, including low birth weight, preterm birth, and infections.

Mitochondria, double-membraned intracellular organelles, possess about 2–10 copies of their circular genome, called mitochondrial DNA (mtDNA). These organelles are crucial as they are the most important

* Corresponding author at: Centre for Environmental Sciences, Hasselt University, Agoralaan gebouw D, BE-3590 Diepenbeek, Belgium.
E-mail address: dries.martens@uhasselt.be (D.S. Martens).
[1] Authors contributed equally.

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source of reactive oxygen species (ROS) (Mikhed et al., 2015) and responsible for oxidative stress management (Lee and Wei, 2000). While ROS is necessary for cellular signaling (Dröge, 2002), mtDNA is particularly vulnerable to uncontrolled overproduction of ROS associated with oxidative stress due to the lack of protective strategies, such as adequate repair capacity and protective histones (Lee and Wei, 2000; Janssen et al., 2012; Lax et al., 2011). Persistent mitochondrial DNA damage results in mutations in the mitochondrial genome (Hollensworth et al., 2000), leading to further mitochondrial dysfunction and may decrease mtDNA content (mtDNAc) (Malik and Czajka, 2013). Consequently, this can result in multifactorial diseases (Dolcini et al., 2020; Duarte-Hospital et al., 2021; Reddam et al., 2022; Smith et al., 2023); including breast cancer (Xia et al., 2009), type 2 diabetes (Wong et al., 2009; Gianotti et al., 2008; Choi et al., 2001), and dementia (Phillips et al., 2014; Srivastava, 2017).

Animal studies have found associations between exposure to heat and cold temperature stress and uncontrolled overproduction of ROS, causing oxidative stress. Consequently, damage to mitochondrial DNA, proteins, and lipids occurs, affecting mitochondrial respiration, integrity, and function (Hsu et al., 1995; Qian et al., 2004; Chen et al., 2020; Blagojevic, 2011; Marquez-Acevedo et al., 2023). However, limited evidence exists on the association between temperature exposure and human mtDNAc. A study in an elderly population showed higher mitochondrial DNA copy number (mtDNAcn) correlated with lower odds of cognitive decline with decreasing temperature than those with lower mtDNAcn (Dolcini et al., 2020). Peng et al. (2017) observed increased mitochondrial oxidative damage with short-term increases in mean air temperature during summer in older individuals from the Normative Aging Study (Peng et al., 2017). Positive associations between increased mean air temperature and mtDNA lesions were observed in winter.

In early life, mtDNAc is crucial in mitochondrial function, cell growth, and morphology (Jeng et al., 2008). Consequently, alterations in mtDNAc have been linked to pregnancy-related complications causing developmental delay and increased susceptibility to other neonatal diseases (Perez et al., 2019). However, whether prenatal ambient temperature exposures may relate to alterations in the mtDNAc of the newborn is currently unknown.

Therefore, we hypothesized that *in utero* exposure to low and high temperatures are associated with differences in newborn mtDNAc at birth. To test the hypothesis, we studied the association of prenatal ambient temperature exposure with umbilical cord blood and placental mtDNAc within the ENVIR*ON*AGE (ENVIRonmental influence ON early AGEing) birth cohort.

2. Methods

2.1. Study population and data collection

This study included 1,275 mother-newborn pairs (all singleton births with $\geq$ 36 weeks of gestation) with mtDNAc measurements available in both cord blood and placenta and a complete history of exposure data from the ongoing population-based ENVIR*ON*AGE birth cohort. Mother-newborn pairs were recruited between February 2010 and July 2017 in the delivery room of the East-Limburg Hospital in Genk (Belgium). Detailed information about the study procedures has been described previously (Janssen et al., 2017). Our study complies with the Helsinki Declaration and is approved by the ethics committees of Hasselt University and East-Limburg Hospital in Genk (reference no. B371201216090). To obtain a population having complete data (n = 911) on the potential confounders for statistical analysis, we excluded 364 mother-newborn pairs due to missing education data (n = 17), lack of smoking data (n = 8), and lack of blood cell count measurements (n = 339). A complete overview of the study population inclusion is given in Supplementary Fig. S1. A comparison between the general characteristics of the analyzed study population and the not-analyzed fraction of the mother-newborn pairs in the cohort is provided in Supplementary Table S1.

During the first antenatal visit (weeks 7–9 of gestation), the gestational age was estimated based on the mother's last menstrual period and an ultrasonography examination. Upon arrival in the hospital for delivery, written informed consent was given by all participating mothers, and study questionnaires about their health and lifestyle were completed. Mothers provided information about their age, educational status, smoking status, and residential address. Maternal education was categorized following the ISCED guidelines (UNESCO, 2011) as "low" (no diploma or primary school degree), "middle" (secondary school degree), or "high" (college or university degree). Mothers' smoking status during pregnancy was coded as "never smoker", "former smoker" when they stopped smoking before their pregnancy, or "smoker" when they continued during pregnancy. Perinatal parameters such as date of birth, newborn sex, and birth weight were obtained from the medical record.

2.2. Sample collection and DNA extraction

Immediately after delivery, umbilical cord blood was collected in BD Vacutainer® plastic whole-blood tubes with spray-coated K2EDTA (BD, Franklin, Lakes, NJ, United States). After collection, samples were centrifuged at 3,200 rpm for 15 min to separate the buffy coat and immediately frozen at −80 °C. A separate K2EDTA tube was collected for blood cell counts (including platelet counts) and differential leukocyte counts. This was obtained using an automated cell counter with flow differential (Cell Dyn 3500; Abbott Diagnostics, Abott Park, IL, USA). Placentas were collected in the delivery room and deep-frozen at −20 °C within 10 min after delivery. Following a standardized procedure outlined by Adibi et al. (2010) and previously described elsewhere (Janssen et al., 2017; Adibi et al., 2010), we defrosted the placentas to collect tissue samples for DNA extraction. Briefly, four different placental biopsies (1–2 cm^3) were taken 1–1.5 cm below the chorioamniotic membrane at standardized sites from the fetal-faced side at ~4cm from the umbilical cord and stored at −80 °C. Only one biopsy was used for placental mtDNAc measurement. Care was taken by visual examination and dissection to avoid the chorioamniotic membrane contamination.

Following the manufacturer's instructions, DNA was extracted from cord blood buffy coat and placental tissue using the QIAamp DNA Mini Kit (Qiagen Inc., Venlo, the Netherlands). The extracted DNA concentration and purity were measured using the Nanodrop spectrophotometer (ND-1000; Isogen Life Science, De Meern, the Netherlands). DNA integrity was assessed using gel electrophoresis (1.5 % agarose gel). The extracted DNA was stored at −80 °C until it was needed for further analysis.

2.3. Measurement of mtDNA content

We used a quantitative real-time polymerase chain reaction (qPCR) assay to measure mtDNAc in cord blood leukocytes and placental tissue, as described previously elsewhere (Janssen et al., 2012; Janssen et al., 2017). This procedure involved determining the ratio of two mitochondrial gene copy numbers (MTF3212/R3319 and MT-ND1) to two single-copy nuclear control genes (RPLP0 and ACTB). Primer sequences for the selected genes and their accession number are provided in Supplementary Table S2. Before qPCR, the extracted genomic DNA was diluted to a final concentration of 2 ng/μL in RNase-free water, and this concentration was evaluated using Quant-iT™ PicoGreen® (Life Technologies, Europe). In total, 5 ng DNA was used for the qPCR reactions, mixed with Fast SYBR® Green I dye 2X (Applied Biosystems) and 300 nM forward and reverse primer. All reactions were performed in triplicate in a MicroAmp® Fast Optical 384-Well Reaction Plate on a 7900HT Fast Real-Time PCR System (Applied Biosystems). To account for inter-run variability, six inter-run calibrators were analysed on each reaction

plate, in addition, three no template controls were added to detect possible DNA contamination. The thermal cycling profile was as follows: 20 s at 95 °C for activation of the AmpliTaq Gold® DNA polymerase, followed by 40 cycles of 1 s at 95 °C for denaturation and 20 s at 60 °C for annealing/extension. Melting curve analyses were used at the end of each run to confirm the specificity of the reaction and the absence of primer dimers. The average mtDNAc was calculated using the qBase software (Biogazelle, Zwijnaarde, Belgium). mtDNAc is calculated as a calibrated normalized relative quantity (CNRQ). The latter is achieved by first calculating the RQ based on the delta-Cq method for two mitochondrial (M) and two single-copy gene (S) obtained Cq values. As the choice of a calibrator sample (sample to which subsequent normalization is performed, delta-delta-Cq) strongly influences the error on the final relative quantities (as a result of the measurement error on the calibrator sample), normalization is performed to the arithmetic mean quantification values for all analyzed samples, which results in the NRQ. Samples are measured over different qPCR plates. Therefore, six IRCs are used to calculate an additional correction factor to eliminate run-to-run differences, resulting in the final mtDNAc ratio (CNRQ). Mathematical calculation formulas to obtain RQ, NRQ, and CNRQs were provided (Hellemans et al., 2007). Cord blood mtDNAc was measured in four batches, and placental mtDNAc was measured in five batches.

2.4. Exposure assessment

The Belgian Royal Meteorological Institute provided the daily mean temperature (°C) at a representative measuring station in Diepenbeek (Belgium). This measuring station is representative of participants whose average distance from the station (5th to 95th percentiles) was 14.7 km (0.6 to 81 km) and is the closest to the recruitment hospital (~ 6.6 km). To explore potential critical exposure windows during pregnancy, the average daily mean temperature over each week was calculated backward from the date of birth. Lagged weekly temperature exposures were obtained for the first week until the 36th week before birth, as complete exposure data was required for distributed lag nonlinear modelling (DLNMs) (Distributed et al., 2011). Newborns with a gestational age of less than 36 weeks were excluded.

Exposure to ambient air pollution [particulate matter with aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$)] is associated with newborn mtDNAc and can vary daily due to meteorological conditions (Pérez et al., 2020). For this reason, we considered ambient air pollution as a potential confounder (Janssen et al., 2012; Rosa et al., 2017; Van Der Stukken et al., 2023; Hu et al., 2020). Based on the maternal residential address, we modeled daily mean $PM_{2.5}$ concentrations (in $\mu g/m^3$) during pregnancy using a high-resolution spatial–temporal interpolation method (kriging) (Janssen et al., 2008) in combination with a dispersion model (Lefebvre et al., 2013; Lefebvre et al., 2011), as described previously (Janssen et al., 2017). This method combines cover data from satellite images (CORINE land cover dataset) and air pollution data from fixed-site monitoring stations. This model chain provides interpolated air pollution values on a dense, irregular receptor point grid coupled with a dispersion model that uses emissions from point and line sources (Lefebvre et al., 2013; Lefebvre et al., 2011). More than 80 % ($R^2 = 0.8$) of the temporal and spatial variability in $PM_{2.5}$ concentrations in the Flemish region of Belgium was explained by this interpolation tool (Maiheu et al., 2012). As with weekly mean temperature exposures, the weekly mean exposure to ambient $PM_{2.5}$ was calculated for 1 to 36 weeks before birth, backwards from the date of birth. Address changes of mothers during pregnancy were considered (n = 101; 11.08 % of the study population).

2.5. Statistical analysis

R version 4.2.2 (R Core Team, Vienna, Austria) was used for all data management and statistical analyses. To improve normal distribution, mtDNAc was log_{10}-transformed. To check model assumptions, we evaluated residual plots of the regression models, and a log_{10}-transformation improved normality on the residuals.

Distributed lag nonlinear models (DLNMs) were fitted using the dlnm package (Distributed et al., 2011) to model the association between log_{10}-transformed newborn mtDNAc and average weekly mean temperature, accounting for the period from birth (lag = 0) backwards until 36 weeks before birth (lag = 36 weeks). A DLNM is built on a cross-basis of lagged exposures, which allows the simultaneous estimation of the exposure–response association and the lag–response association (Gasparrini, 2014). We modelled the association along the lag dimension (1–36 gestational weeks before birth) using a natural cubic spline with 5 degrees of freedom (df) using five equally spaced knots. The association along the exposure dimension was first modelled similarly using a natural cubic spline with df = 5, and the median weekly average temperature (11.0 °C) was used as the reference value to estimate the temperature effect. The estimated temperature effect showed a relatively flat pattern at average temperatures and was only noticeable at both ends of the temperature distribution (Supplemental Fig. S2). To address this, we applied a double-threshold DLNM in the subsequent analysis. A double-threshold DLNM implies no association between temperature and the outcome for temperatures between the high and low temperature thresholds, whereas the association between temperature and the outcome is assumed to be linear above the high and below the low temperature thresholds. We evaluated multiple predefined high temperature thresholds: 17.3 °C (85th percentile), 18.4 °C (90th percentile), and 19.8 °C (95th percentile). As low temperature thresholds, we used 0.8 °C (5th percentile), 2.8 °C (10th percentile), and 4.2 °C (15th percentile). The main model was adjusted for *a priori*-selected covariates and variables with a possible link with ambient temperature and mtDNAc: newborn sex, birth weight, gestational age, month of delivery, maternal age, smoking status during pregnancy, education, and batch of mtDNAc measurement. To account for the potential confounding effect of $PM_{2.5}$ exposure in the same period, we additionally adjusted for weekly mean $PM_{2.5}$ exposure by including a $PM_{2.5}$ cross-basis with a linear function along the exposure dimension and natural cubic splines (df = 5, equally spaced knots) along the lag dimension (Martens et al., 2017). In addition, the cord blood mtDNAc model was adjusted for cell type compositions of neutrophils, lymphocytes, monocytes, eosinophils, and platelets to account for cord blood cell distribution.

The temperature effects were estimated as the percent (%) difference (calculated as $(10^{(\beta)}\text{-}1)$ X 100 %) with 95 % CI (calculated as $(10^{(\beta \pm 1.96SE)}\text{-}1)$ X 100 %) in mtDNAc for a 1 °C increase in temperature above the high temperature thresholds, as the high temperature effect, and a % difference in mtDNAc for a 1 °C decrease below the low temperature thresholds, as the low temperature effect. Finally, the overall cumulative effect over the entire pregnancy and each trimester of pregnancy was calculated. The significance level of 0.05 was applied, and 95 % confidence intervals were shown for each estimate.

We conducted sensitivity analyses focusing on the significant weeks identified in the initial findings and tested the robustness of the observed cumulative effects during the different gestational periods (trimesters and entire pregnancy) using the 90th percentile high temperature threshold and 10th percentile low temperature threshold. We assessed the association while excluding the cross-basis for $PM_{2.5}$ or additionally adjusting for maternal prepregnancy body mass index (BMI), maternal fruit and vegetable intake, physical activity during pregnancy, and maternal plasma and cord blood plasma vitamin D levels, respectively. We compared the cumulative effect estimates in the different sensitivity analyses.

3. Results

3.1. Study population characteristics

General characteristics of the 911 mother-newborn pairs in the study

population are provided in Table 1. The newborns, which included 457 (50.2 %) boys, had a mean [standard deviation (SD)] gestational age of 39.7 (1.2) weeks and a mean (SD) birth weight of 3,439 (439) g. Mothers had a mean (SD) age of 29.6 (4.6) years, ranging from 17 to 46 years, and a prepregnancy BMI of 24.6 (4.8) kg/m^2. Most mothers had a college or university degree (53.0 %) and never smoked (64.8 %). MtDNAc determined in cord blood and placental tissue cells ranged from 0.2 to 6.3 and from 0.2 to 12.5, respectively. Cord blood mtDNAc and placental mtDNAc were not correlated (r = −0.003; p = 0.8). The average weekly mean (5th to 95th percentile) ambient temperature during pregnancy was 10.8 °C (7.4 to 13.8), and the average weekly mean (5th to 95th percentile) residential $PM_{2.5}$ exposure was 12.8 μg/m^3 (8.9 to17.3 μg/m^3). Prenatal temperature and $PM_{2.5}$ characteristics for the included mother-newborn pairs are shown in Supplementary Table S3.

Table 1
Mother-newborn characteristics (n = 911) of the included ENVIRONAGE participants.

Characteristics	Mean ± SD or n (%)
Newborns	
Birth weight, g	3.439 ± 439
Sex	
Male	457 (50.2)
Female	454 (49.8)
Gestational age (weeks)	39.7 ± 1.2
Month of delivery	
Dec-Feb	214 (23.5)
Mar-May	236 (25.9)
Jun-Aug	218 (23.9)
Sep-Nov	243 (26.7)
Cell count	
Neutrophils (%)	8.0 ± 3.0
Lymphocytes (x 10.E3/μL)	4.8 ± 1.8
Monocytes (%)	1.4 ± 0.6
Eosinophils (%)	0.5 ± 0.3
Platelets (x 10.E3/μL)	263.7 ± 83.1
Plasma vitamin D, ng/mL[a]	24.6 ± 12.8
Mothers	
Age (years)	29.6 ± 4.6
Prepregnancy BMI (kg/m^2)	24.6 ± 4.8
Education	
Low	106 (11.6)
Middle	322 (35.4)
High	483 (53.0)
Smoking status	
Never smoker	590 (64.8)
Former smoker	209 (22.9)
Smoker	112 (12.3)
Physical activity[b]	
Low	273
Middle	175
High	422
Fruit and vegetables intake[c]	
< 1 portion/day	108
1 portion/day	280
2 portions/day	323
3 portions/day	168
Plasma vitamin D, ng/mL[d]	18.0 ± 9.2
Weekly mean temperature, °C[e]	10.8 (7.4 to 13.8)
Weekly mean $PM_{2.5}$ exposure, μg/m^3[e]	12.8 (8.9 to 17.3)

SD, standard deviation; BMI, body mass index; $PM_{2.5}$, particulate matter with aerodynamic diameter ≤ 2.5 μm.

[a] Measured in cord blood EDTA plasma, data missing on n = 62.

[b] Data missing on n = 41 and was defined as low when active (at least 20 min) less than once a week, middle when active once a week, and high when active two or more times a week during pregnancy.

[c] Data missing on n = 32.

[d] Measured in maternal EDTA plasma, data missing on n = 124.

[e] Presented as weekly means (5th to 95th percentiles) and for 36 weeks of gestation.

3.2. Association between prenatal ambient temperature exposure and mtDNA content in newborns

As we observed only effects at the extremes of the temperature range, we applied a double threshold model to evaluate further detailed high and low ambient temperature effects on mtDNAc (Supplementary Fig. S2). We used the 5th, 10th, and 15th percentile of the temperature distribution as low temperature thresholds. Alternatively, the 85th, 90th, and 95th percentile of the temperature distribution were used as high temperature thresholds.

3.2.1. High temperature effects on mtDNA content

Lag-specific (weekly) DLNM estimates of the high temperature effects on cord blood and placental mtDNAc are presented in Fig. 1.

For cord blood mtDNAc, a 1 °C increase in weekly mean ambient temperature above the high temperature threshold of 17.3 °C (85th percentile) was associated with lower mtDNAc from weeks 13 to 25 before birth (Fig. 1A, Supplementary Table S4). The high temperature effect was the strongest at the 19th week before birth, where a 1 °C increase in weekly mean ambient temperature was associated with −2.23 % lower mtDNAc (95 % CI: −3.68, −0.76). Among similar lines, when applying a more stringent high temperature threshold of 18.4 °C (90th percentile), cord blood mtDNAc was negatively associated with ambient temperatures from 14 to 24 weeks before birth (Fig. 1B, Supplementary Table S4). No significant associations were found using the most extreme high temperature threshold of 19.8 °C (95th percentile) (Fig. 1C, Supplementary Table S4). When evaluating overall cumulative high temperature effects using the different high temperature thresholds (Supplementary Table S5), each 1 °C increase in ambient temperature above the high temperature threshold of 17.3 °C (85th percentile) and 18.4 °C (90th percentile) during trimester 2 was associated with −21.35 % (95 % CI: –32.96, −7.73) and −25.56 % (95 % CI: −39.82, −7.92) lower mtDNAc, respectively. A 1 °C increase in ambient temperature for the entire pregnancy above the same thresholds resulted respectively in −36.01 % (95 % CI: −56.73, −5.36) and −43.14 % (95 % CI: −66.02, −4.84) lower mtDNAc.

For placental mtDNAc, an increase in weekly ambient temperature above the high temperature threshold of 17.3 °C (85th percentile) was associated with higher placental mtDNAc from 16 to 21 weeks before birth (Fig. 1D, Supplementary Table S4). When applying a stringent high temperature threshold of 18.4 °C (90th percentile), a negative association with mtDNAc was observed from 27 to 33 weeks before birth (Fig. 1E, Supplementary Table S4). Further applying a stricter high temperature threshold of 19.8 °C (95th percentile) showed a similar negative association with placental mtDNAc between 28 and 33 weeks before birth and revealed strong positive associations with an increase in temperature above the high temperature threshold between weeks 1 and 20 before birth (Fig. 1F, Supplementary Table S4). The overall cumulative high temperature effects are shown in Supplementary Table S5. Considering the high temperature threshold of 17.3 °C (85th percentile), a significant positive association was observed during trimester 2, resulting in 19.09 % (95 % CI: 0.65, 40.90) higher mtDNAc. An inverse association was observed during trimester 1 of pregnancy when applying the 18.4 °C and 19.8 °C high temperature thresholds, resulting in −26.13 % (95 % CI: −40.78, −7.85) and –33.19 % (95 % CI: −53.39, −4.24) lower mtDNAc, respectively. Applying the strictest high temperature threshold of 19.8 °C showed a positive association over all the other pregnancy periods.

3.2.2. Low temperature effects on mtDNA content

Lag-specific (weekly) DLNM estimates of the low temperature effects in cord blood and placental mtDNAc are presented in Fig. 2. A decrease in ambient temperature below the low temperature threshold of 4.2 °C (15th percentile) was weakly associated with higher cord blood mtDNAc from 20 to 23 weeks before birth (Fig. 2A, Supplementary Table S6). We observed significant positive low temperature effects at the low

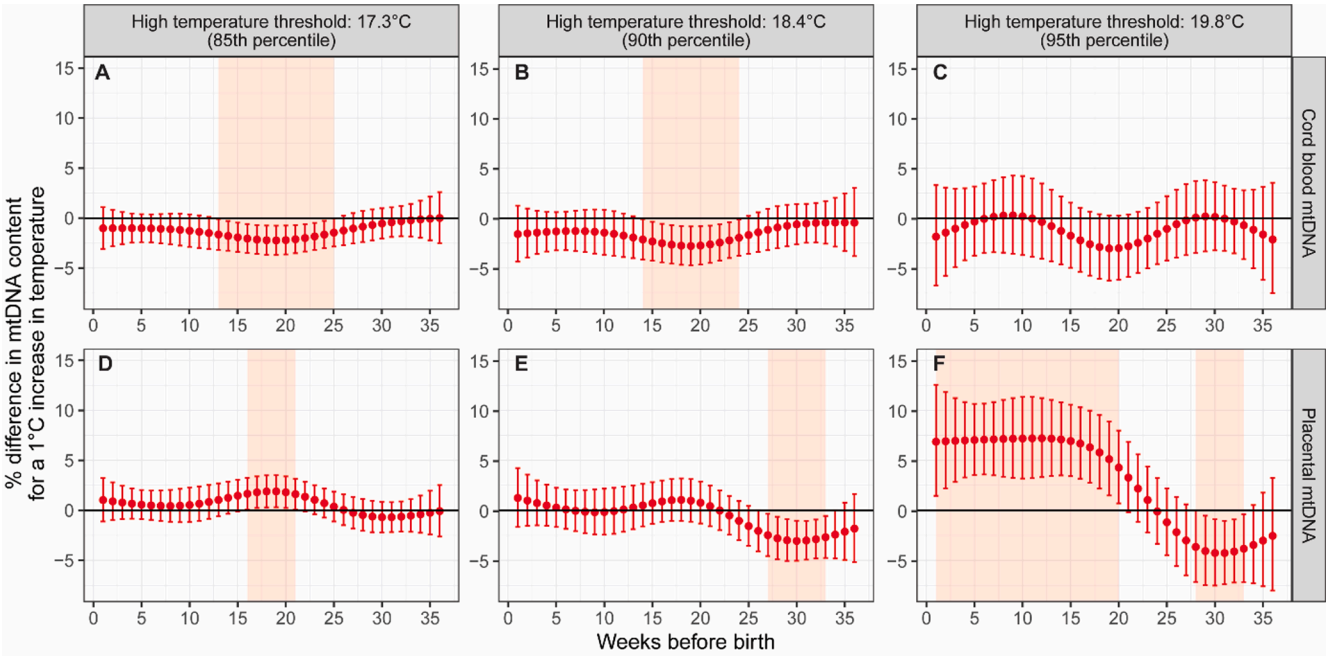

Fig. 1. High temperature effect estimates on cord blood and placental mtDNAc. Week-specific estimates are provided as percent differences (%) in cord blood (A, B, C) and placental (D, E, F) mtDNAc [with 95 % confidence interval (CI)] for a 1 °C increase in ambient temperature above the high temperature thresholds for associations with high temperatures. From left to right, the high temperature thresholds are 17.3 °C, 18.4 °C, and 19.8 °C, corresponding to the 85th, 90th, and 95th percentiles of the temperature distribution, respectively. The red shaded areas represent the identified significant sensitive windows. Models were adjusted for birth weight, gestational and maternal age, newborn sex, month of delivery, maternal smoking status, maternal education, batch, and maternal particulate matter with aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$) exposure. Additionally, cord blood models were adjusted for blood cell count (neutrophils, lymphocytes, monocytes, eosinophils, and platelets). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

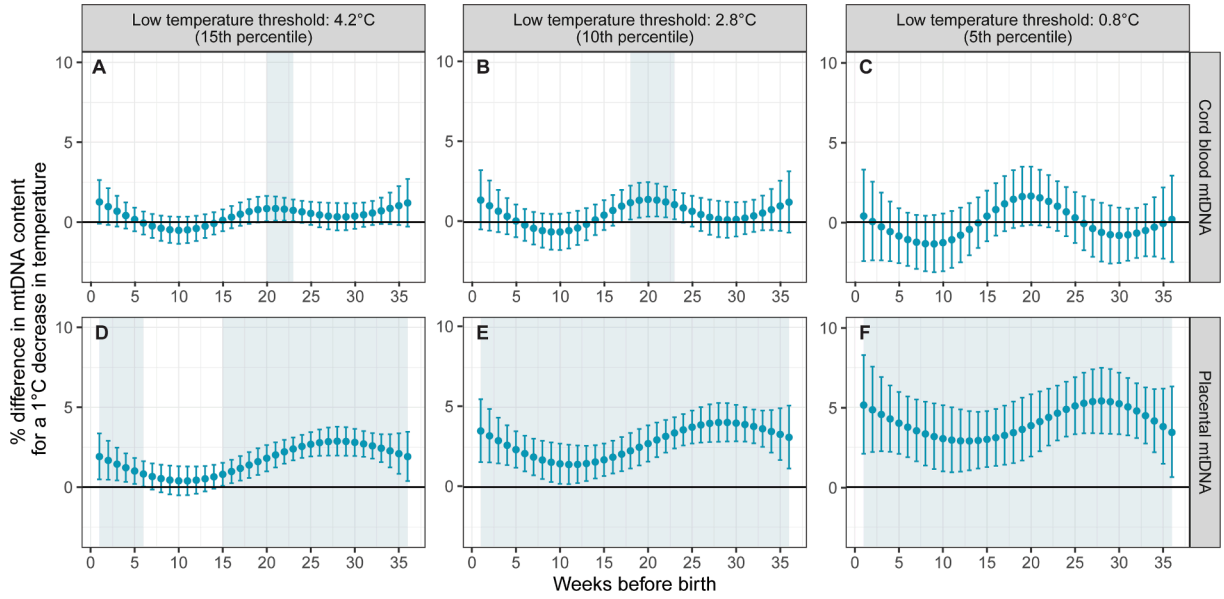

Fig. 2. Low temperature effect estimates on cord blood and placental mtDNAc. Week-specific estimates are provided as percent differences (%) in cord blood (A, B, C) and placental (D, E, F) mtDNAc [with 95 % confidence interval (CI)] for a 1 °C decrease in ambient temperature below the low temperature thresholds for associations with low temperatures. From left to right, the low temperature thresholds were 4.2 °C, 2.8 °C, and 0.8 °C, corresponding to the 15th, 10th, and 5th percentiles of the temperature distribution, respectively. The blue shaded areas represent the identified significant sensitive windows. Models were adjusted for birth weight, gestational and maternal age, newborn sex, month of delivery, maternal smoking status, maternal education, batch, and maternal particulate matter with aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$) exposure. Additionally, cord blood models were adjusted for blood cell count (neutrophils, lymphocytes, monocytes, eosinophils, and platelets). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

temperature threshold of 2.8 °C (10th percentile) in weeks 18 to 23 before birth (Fig. 2B, Supplementary Table S6). No low temperature effects were observed for cord blood mtDNAc using the most stringent 0.8 °C (5th percentile) low temperature threshold (Fig. 2C, Supplementary Table S6). Only an overall cumulative low temperature effect over trimester 2 of pregnancy was observed for cord blood mtDNAc

when applying the 2.8 °C threshold. Each 1 °C decrease in ambient temperature below the low temperature threshold was associated with 11.33 % (95 % CI: 0.69, 23.09) higher cord blood mtDNAc (Supplementary Table S7).

For placental mtDNAc, a decrease in weekly ambient temperature below all applied low temperature thresholds was associated with higher placental mtDNAc (Fig. 2 D, E, and F, Supplementary Table S6). At the low temperature threshold of 4.2 °C (15th percentile), we identified 1 to 6 weeks and 15 to 36 weeks before birth as sensitive windows (Fig. 2D, Supplementary Table S6). When applying the more stringent low temperature thresholds of 2.8 °C (10th percentile) (Fig. 2E, Supplementary Table S6) and 0.8 °C (5th percentile) (Fig. 2F), ambient exposure below the threshold was associated with higher mtDNAc in all gestational weeks. Cumulative low temperature effects consistently showed significant positive associations for all applied thresholds and over all the different pregnancy periods (Supplementary Table S7).

3.3. Sensitivity analyses

We tested the robustness of our findings with several sensitivity analyses (Supplementary Table S8 and S9). For the observed high temperature effects in cord blood and placenta mtDNAc, our results remained robust after additional adjustments to prepregnancy BMI, maternal fruit and vegetable intake, physical activity during pregnancy, and maternal plasma and cord blood plasma vitamin D levels. Removing $PM_{2.5}$ adjustment from the model slightly weakened the cumulative effects (Supplementary Table S8).

All sensitivity analyses were robust for the observed low temperature effects in cord blood and placenta (Supplementary Table S9).

4. Discussion

We aimed to identify critical gestational windows of exposure to low and high temperatures associated with newborn mtDNAc. Our findings indicated that ambient temperature exposure is associated with mtDNAc in cord blood and placental tissue at birth. The key findings were that *a*) prenatal temperature exposure above the high temperature threshold of 18.4 °C (90th percentile) was associated with lower cord blood and placental mtDNAc; *b*) for placental mtDNAc, when we applied the strictest high temperature threshold of 19.8 °C (95th percentile), it showed a strong positive association over all the pregnancy periods, except for trimester 1; *c*) prenatal temperature exposure below the low temperature threshold of 2.8 °C (10th percentile) and 4.2 °C (15th percentile) was associated with higher cord blood mtDNAc; *d*) low temperature effects were more pronounced in placental tissue resulting in higher mtDNAc associated with decreasing temperatures below all the different applied low temperature thresholds. These results persisted with adjustment for birth weight, gestational and maternal age, newborn's sex, month of delivery, maternal smoking status, maternal education, maternal particulate matter with aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$) exposure, batch of mtDNA measurement, and blood cell counts. To our knowledge, this is the first study investigating the association between prenatal ambient temperature exposure and mtDNAc at birth.

Previous research has shown that extreme ambient temperatures during pregnancy can disrupt maternal thermal homeostasis as the ability of pregnant women to thermoregulate is compromised (Wells et al., 2002). Adverse birth outcomes, such as an increased risk for preterm birth, stillbirth, and low birth weight associated with exposure to high and low ambient temperatures, have already been reported by several studies (Chersich et al., 2020; Ruan et al., 2023). However, the biological mechanisms underlying these effects are poorly understood.

Mitochondria are intracellular organelles playing an essential role as central regulators of metabolism and oxidative stress but are particularly vulnerable to uncontrolled overproduction of ROS associated with oxidative stress (Lee and Wei, 2000; Janssen et al., 2012; Lax et al., 2011). ROS include both radical and non-radical oxygen molecules such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•), which are produced through partial reduction of oxygen. These ROS are produced endogenously during mitochondrial oxidative phosphorylation or can originate from exogenous sources such as interactions with xenobiotic compounds. Oxidative stress occurs when ROS surpass the cellular antioxidant defense capacity due to increased ROS production or decreased antioxidant capacity. Environmental exposures, including higher ambient temperatures, often enhance biological activities that trigger ROS production, producing cellular oxidative stress (Ray et al., 2012).

However, research on the effect of ambient temperature exposure on human mtDNAc remains limited. For cord blood mtDNAc, we found that prenatal temperature exposure above 18.4 °C (90th percentile) during trimester 2 was linked to lower cord blood mtDNAc. Conversely, temperature exposure below 2.8 °C (10th percentile) was associated with higher cord blood mtDNAc. Similar results were found in another study of the ENVIR*ON*AGE birth cohort, where higher prenatal ambient temperature exposure above 19.5 °C during gestational weeks 14–36 was associated with shorter cord blood telomere length (TL). A decrease in ambient temperature (below 5.0 °C) was associated with longer cord blood TL (Martens et al., 2019). This aligns with our findings for cord blood mtDNAc, as previous studies have shown a positive association between mtDNAc and TL (Tyrka et al., 2015; Kim et al., 2013; Qiu et al., 2015; Melicher et al., 2018). Both mtDNAc and TL are considered surrogates for personal health outcomes and aging processes (Hang et al., 2018). Additionally, a study of 1207 Chinese adults found significantly higher blood mtDNAc in colder northern climates compared to warmer southern regions (Cheng et al., 2013) and one study linked short-term temperature increases to blood mitochondrial oxidative damage and mtDNA lesions (Peng et al., 2017). These findings align with our results, which show higher cord blood mtDNAc for lower temperatures and lower cord blood mtDNAc for exposure to higher temperatures.

Placental mtDNAc showed a strong positive association with ambient temperature exposure during pregnancy when we applied the strictest high temperature threshold of 19.8 °C (95th percentile), except for trimester 1. Low temperature exposure was linked to higher placental mtDNAc during the whole gestation and for all applied thresholds. This aligns with a previous study within the ENVIR*ON*AGE birth cohort, which found that a decrease in ambient temperature (below 5.0 °C) was associated with longer placental TL (Martens et al., 2019). Although some of our findings are consistent with prior research, it is important to distinguish between cord blood and placental responses when interpreting the impact of environmental exposures on mtDNAc. To fully understand the impact of ambient temperature exposure on mtDNAc and health and diseases later in life, further research investigating underlying biological mechanisms is needed.

Studies on heat stress in animals indicate that mitochondria are among the primary cellular structures affected by heat stress (Hsu et al., 1995; Qian et al., 2004; Cheng et al., 2013). In mammalian oocytes, heat stress has been shown to lower mitochondrial activity and impair embryo development (Sakatani et al., 2008; Chen et al., 2016). The biological mechanism stated in these studies suggests that embryonic development seems to be correlated with mitochondrial activity in oocytes, characterized by mitochondrial membrane potential ($\Delta\Psi$). $\Delta\Psi$ in oocytes is highly responsive to the environment, particularly under stressful conditions (Slimen et al., 2014). Variations in $\Delta\Psi$ seem directly related to signal transduction, Ca++ homeostasis, and cell apoptosis (Clay Montier et al., 2009). Exposure to heat stress induces ROS overproduction, causing cell apoptosis and/or reduced mitochondrial membrane potential, and may decrease mtDNAc (Golden et al., 2001; Hou et al., 2010; Lamson and Plaza, 2002; Sahin et al., 2011; Lee and Wei, 2005). These effects may contribute to an increased risk of metabolic disorders and reduced performance in animals under heat stress (Cosemans et al., 2023). However, other studies propose that persistent or heightened oxidative stress can alter the rate of mtDNA replication,

resulting in compensation mechanisms for these mutations by increasing their number and replication rate and, therefore, leading to alterations in mtDNAc and function (Maheshwari et al., 2022; May-Panloup et al., 2005; Steuerwald et al., 2000; Wakefield et al., 2011; Van Blerkom et al., 1995; Ge et al., 2012). They postulate that when oxidative damage occurs, an increase in mtDNA occurs as a compensatory mechanism to counteract the presence of defective mitochondria with impaired respiratory chains or mutated mtDNA. This increase in mtDNA aims to restore mitochondrial function (Steuerwald et al., 2000; Ge et al., 2012; Trifunovic et al., 2004).

In early life, mitochondria are crucial for embryonic and neonatal development, enabling nutrient oxidation and maintaining calcium (Ca++) homeostasis (Soto and Smith, 2009; Nabenishi et al., 2012; Jones, 2004). Studies in animals with mtDNA mutations have shown abnormal embryonic development and growth restriction of both the fetus and placenta (Van Blerkom, 2011; Hansen, 2009; Ozawa et al., 2002; Sakatani et al., 2004). Postnatally, adequate mitochondrial energy metabolism is essential for neonates to support their rapid growth and adaptation to extrauterine life. Neonatal organs, such as the heart and brain, primarily rely on aerobic metabolism, which depends on optimal mitochondrial function (Soto and Smith, 2009).

Trimester 2 emerged as a potential sensitive exposure window for both high and low temperature exposure affecting cord blood mtDNAc. While no prior studies address prenatal temperature exposure and mtDNAc, research on other prenatal environmental exposures on mtDNAc and TL supports similar sensitive windows (Mishra et al., 2024). For instance, prenatal $PM_{2.5}$ exposure during 25–32 weeks and PM_{10} exposure during 25–31 weeks of pregnancy were significantly associated with lower cord blood mtDNAc (Hu et al., 2020). Another study observed shorter cord blood TL associated with $PM_{2.5}$ exposure during midgestation (weeks 12–25) (Martens et al., 2017). These windows align with critical fetal developmental stages, including rapid fetal growth, organogenesis, a burst of ROS production and heightened mitochondrial activity, which are necessary to meet increased metabolic demands, potentially making the fetus more vulnerable to environmental stressors during this stage. Placental mtDNAc showed a strong positive association with ambient temperature exposure during pregnancy when we applied the strictest high temperature threshold of 19.8 °C (95th percentile), except for trimester 1. These results might reflect a potential protective capacity of the placenta to sustain fetal nutrient supply and fetal protection to thermal stress. Furthermore, the syncytiotrophoblast layer of the placenta becomes thinner with gestational age, and fetal capillary development increases causing enhanced maternal-fetal exchange of nutrients and particles including potentially harmful pollutants (Proietti et al., 2013).

The fact that we observed different responses for umbilical cord blood and placental mtDNAc demands consideration and may reflect distinct biological roles and susceptibilities of these tissues to environmental exposures. In general, placental mtDNAc showed higher mtDNAc in response to cold stressors and extreme high temperature stressors, while cord blood mtDNAc tended to lower under both low and high temperature stressors. The placental mtDNAc effect sizes were also higher, potentially indicating a protective role buffering temperature effects on cord blood mtDNAc, which had smaller effect sizes. These differences might partly result from the placental tissue consisting of fetal and maternal cells, whereas umbilical cord blood originates solely from the fetus. The mixed cellular composition of placental tissue may introduce complexities and potential uncertainties in its genomic or epigenomic analyses (Patel et al., 2014). It is also important to note that umbilical cord blood possesses a separate circulation system that may not accurately reflect the conditions in other tissues (Gemma et al., 2006). Platelet counts, which can elevate mtDNAc without affecting nuclear DNA, may also influence results (Banas et al., 2004; Cossarizza et al., 2003). However, we accounted for this by adjusting cord blood mtDNAc models for blood cell counts, including platelet counts. Finally, variations in the turnover rates of mtDNA among different tissues have been observed, which could also contribute to disparate effects on cord blood and placental tissue (Collins et al., 2003; Gross et al., 1969).

The DOHaD Theory underscores the importance of our findings as fetal development is a crucial period in life and might predict an individual's susceptibility to adult diseases in later life (Barker, 1998). Since fetuses' detoxification system is not fully developed, exposure to environmental pollutants can cause adverse birth outcomes such as preterm birth and low birth weight (Kuehn and McCormick, 2017; Ge et al., 2012; Breton et al., 2019; Goldenberg et al., 2008). Moreover, research showed that mutations in mtDNA are associated with childhood intelligence (Bijnens et al., 2019) and multifactorial diseases (Dolcini et al., 2020; Duarte-Hospital et al., 2021; Reddam et al., 2022; Smith et al., 2023; Schnegelberger et al., 2021); such as breast cancer (Xia et al., 2009), type 2 diabetes (Wong et al., 2009; Gianotti et al., 2008; Choi et al., 2001), diabetic retinopathy (Journal et al., 2017), dementia (Phillips et al., 2014; Srivastava, 2017), maternally inherited diabetes (Adam et al., 2000) and deafness (Ryzhkova et al., 2018).

Our study has several strengths. First, this study was conducted within a large-sample-size cohort of more than 900 newborns. In addition, we adjusted for important potential confounders, including birth weight, gestational and maternal age, newborn sex, month of delivery, smoking status, maternal education, $PM_{2.5}$ exposure, batch of mtDNAc, and blood cell counts. Also, by applying a double-threshold DLNM, we could identify low and high temperature thresholds and critical time windows during pregnancy for the association between ambient temperature exposures and mtDNAc at birth. Finally, our study results are generalizable because our study population represents the gestational segment of the population at large (Janssen et al., 2017).

Some limitations within this study should be kept in mind. In the analyses, we obtained ambient temperature exposure data from a fixed measuring station in Diepenbeek, Belgium. However, this station is representative of participants whose average distance from the station was 14.7 km (0.6 to 81 km) and is the closest to the recruitment hospital (6.6 km); this may introduce potential exposure misclassification. Secondly, this study did not provide more accurate temperature measurements, such as individual exposure to ambient temperature, body temperature, and information on maternal fever during pregnancy as we only used outdoor ambient temperature exposure data. The use of more individualized and accurate temperature measurements in future studies, such as data from wearable devices or indoor sensors, could provide a more accurate picture of actual thermal exposures. It is important to note that pregnant women often spend their time indoors, indoor the temperatures may be different from the ambient temperatures outside because of air conditioning and heating. This was not considered in our study because there was no information available on the amount of time spent indoors or on the use of air conditioning and heating. However, on warm days, outdoor temperature may be a better reflection of the actual exposure, since the proportion of houses equipped with air conditioning in Belgium was relatively low at the moment of sample collection. Although our results were consistent after adjustments, the risk of confounding by possible unknown factors associated with ambient temperature exposure and mtDNAc cannot be excluded. We also acknowledge that the association between prenatal temperature exposure and newborn mtDNAc might depend on the level of $PM_{2.5}$ exposure. However, given the complexity of the analysis based on the exposure cross-basis, and the constraints posed by the statistical power of our study, we were not able to perform an interpretable air-pollution-temperature interaction in the current analysis. Finally, as the exposure to ambient temperatures depends on location, our results may only be generalizable for populations living within climate conditions similar to those of our study population. According to the Köppen-Geiger climate classification (Peel et al., 2007), Belgium falls within the Cfb category, indicating a temperate climate with no dry season, a warm summer, and an oceanic influence (with monthly mean temperatures below 22 °C during the warmest month and above 0 °C during the coldest month). This classification also describes the climates of Western European

countries, parts of southeastern Australia, Tasmania, New Zealand, and North and South America regions. Therefore, different populations may experience higher and lower temperature thresholds, as acclimatization and adaptation responses can differ across diverse climates and populations (Gasparrini et al., 2015). Studying the link between prenatal ambient temperature and mitochondrial DNAc has provided valuable insights that shed light on the intricate relationship between environmental factors and biological processes. The findings from the ENVIRONAGE study highlight the impact of prenatal conditions on mitochondrial health, emphasizing the importance of early-life exposures in shaping long-term health outcomes. However, further longitudinal studies are needed to investigate how altered mtDNAc at birth might influence the development and risk of multifactorial diseases (Gorman et al., 2016; Russell et al., 2020).

5. Conclusion

We showed that ambient temperature exposure is significantly associated with mtDNAc in cord blood and placental tissue. It highlights the importance of understanding the effects and consequences of environmental exposures on susceptible populations, especially pregnant women and fetuses. Our study provides new insights into the possible molecular mechanism underlying adverse birth outcomes and health in later life with exposure to ambient temperatures. The importance of these study findings and further research in this domain gets strengthened by the continuing global warming and climate change worldwide. As global warming and climate change continue, a further in-depth understanding of the underlying mechanisms and potential adverse health consequences associated with mtDNAc later in life is needed.

6. Data sharing

The data supporting this study's findings are available upon request from the corresponding author and after approval by the institutional board.

CRediT authorship contribution statement

Eleni Renaers: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Congrong Wang:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Esmée M. Bijnens:** Writing – review & editing, Methodology, Conceptualization. **Michelle Plusquin:** Writing – review & editing, Methodology, Conceptualization. **Tim S. Nawrot:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Dries S. Martens:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109267.

Data availability

Data will be made available on request.

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