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## Child's buccal cell mitochondrial DNA content modifies the association between heart rate variability and recent air pollution exposure at school

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ARTICLE INFO ABSTRACT Background: Studies investigating short-term exposure to ambient air pollution and heart rate variability (HRV) Handling Editor: Xavier Querol suggest that particulate matter (PM) exposure is associated with reductions in measures of HRV. Mitochondria Keywords: are sensitive to PM exposure and may represent a biologically relevant underlying mechanism. However, evi-Air pollution dence in children is lacking. Mitochondria Objectives: Here we examine whether PM has an influence on children's HRV and evaluate whether mitochon-Heart rate variability drial DNA content (mtDNAc) reflects individual susceptibility. Methods: Within a panel study in primary school children (aged 9–12 years), we measured HRV in a subset of 60 children on three different days during school-time using four indicators: normal-to-normal intervals (SDNN), square root of mean squared difference of normal-to-normal intervals (rMSSD), high frequency (HF), and low frequency (LF). This resulted in a total number of 150 visits (median number of visits per child: 2.5/child). MtDNAc was measured using qPCR in buccal cells. We measured recent PM exposure at the school. Residential 24-hour mean exposure to PM was modelled with a high resolution spatial temporal model. Mixed-effects models were used to estimate the association between HRV and recent PM exposure and potential effect-modification by mtDNAc. Results: Children were on average [SD] 9.9 [1.2] years and comprised 39 girls. Median [25th-75th] recent outdoor  $PM_{2.5}$  and  $PM_{10}$  exposure at school was 6.20 [2.8–12.8] µg/m<sup>3</sup> and 29.3 [24.7–42.0] µg/m<sup>3</sup>, respectively. tively. In children with low mtDNAc (25th percentile), we observed for each  $10 \,\mu g/m^3$  increment in recent PM<sub>2.5</sub> exposure a lowering in the LF parameter with 9.76% (95% CI: -16.9 to -1.99%, p = 0.02;  $p_{int} = 0.007$ ). Children with high mtDNAc did not show this association. For  $PM_{10}$  exposure, we observed an inverse association with three HRV indicators in children with low mtDNAc: -2.24% (95% CI: -4.27 to -0.16%; p = 0.04;  $p_{int} = 0.02$ ) for SDNN, -5.67% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95\% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95\% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95\% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95\% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64% (95\% CI: -10.5 to -0.59%; p = 0.03;  $p_{int} = 0.04$ ) for HF and -6.64\% CI: -10.7 to -2.38%; p = 0.003; p<sub>int</sub> = 0.005) for LF. Conclusions: HRV is inversely associated with recent PM air pollution, especially in children with low mtDNAc. Our data revealed that mtDNAc determines susceptibility to adverse autonomic effects of recent PM exposure in children.

#### 1. Introduction

The autonomic nervous system plays a pivotal role in physiological responses of the cardiovascular system, including blood pressure

regulation, heart rate and vaso-regulatory responses. Aging, stress and cardiovascular risk factors can lead to a deregulation of the autonomic nervous system, which contributes to cardiovascular disease development. Heart rate variability (HRV), or the beat-to-beat alteration in

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*Abbreviations*: PM10, particulate matter with a diameter  $< 10 \,\mu$ m; PM2.5, particulate matter with a diameter  $< 2.5 \,\mu$ M; HRV, heart rate variability; mtDNAc, mitochondrial DNA content; SDNN, Standard deviation of normal-to-normal intervals; rMSSD, the square root of the mean squared difference of normal-to-normal intervals; HF (0.15 to 0.4 Hz), high frequency; LF (0.04 to 0.15 Hz), low frequency

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heart rate, offers a noninvasive indicator of autonomic nervous system activity, with low HRV indicative of reduced parasympathetic cardiac control (Thayer et al., 2010). Both parasympathetic activity (mainly vegetative and restorative functions) and reduced HRV have been associated with immune dysfunction and inflammation, which are involved in the etiology of a range of diseases such as cardiovascular disease, diabetes, Alzheimer's disease, and certain types of cancer (Thayer et al., 2010; Haensel et al., 2008). In children, a reduced HRV state has been observed in children with Attention-Deficit/Hyperactivity Disorder (Rukmani et al., 2016) or overweight/obesity (Birch et al., 2012). Though, there is a lack of literature regarding HRV in healthy children, especially in relation to their environmental exposures.

Exposure to particulate matter (PM) air pollution contributes to cardiovascular morbidity and mortality in adults (Pieters et al., 2012). PM exposure also influences cardiovascular health of children (Pieters et al., 2015; Calderon-Garciduenas et al., 2008; Provost et al., 2017; Zhang et al., 2018) and may cause long-term negative health outcomes. Lower HRV is being considered as part of the pathophysiological mechanism linking PM exposure and cardiovascular effects. Our metaanalysis confirmed that each increase of  $10 \,\mu\text{g/m}^3$  in PM<sub>2.5</sub> (particulate matter with a diameter smaller than 2.5 µm) was associated with 2% significant reductions in the HRV time-domain and frequency-domain measurements. The systematic analysis also brought to the attention that there are no studies in children on PM air pollution and HRV (Pieters et al., 2012). Findings in adults may not generalize to younger individuals, in view of the age-dependent differences in autonomic function (Tanaka et al., 1998; Dietrich et al., 2010; Stolarz et al., 2003) and the fact that children are more vulnerable to the effects of air pollution exposure (Kim, 2004).

The effects of air pollution on HRV have been suggested to be mediated through oxidative stress. Findings from the Normative Aging Study indicated effect modification by glutathione-S-transferase M1 and methylenetetrahydrofolate reductase, and protective effects of statin use, dietary anti-oxidants and methyl nutrients when studying PM-HRV associations (Baccarelli et al., 2008; Schwartz et al., 2005). Mitochondria have a primordial role in energy production and management of oxidative stress (Lee and Wei, 2000). PM exposure has been associated with mitochondrial alterations from early life onwards (Janssen et al., 2012; Zhong et al., 2016). Therefore, markers of mitochondrial function may help to identify inter-individual vulnerability of PM air pollution exposure. In this regard, a study in healthy men showed that the inverse association between PM exposure and HRV outcomes was modified by blood mitochondrial DNA methylation (Byun et al., 2016). In the present study, we hypothesize that HRV in primary school children is associated with recent exposure to PM air pollution, and that mitochondrial function as exemplified by mitochondrial DNA content modulates this association.

#### 2. Methods

#### 2.1. Study population

The COGNAC (COGNition and Air pollution in Children) study enrolled 334 school children between January 2012 and February 2014. The children were 9 to 12 years of age and were recruited at three primary schools in Flanders (Belgium). The study was initially designed to investigate acute effects of air pollution on cognition (Saenen et al., 2016). HRV measurements were implemented in one school during the measuring campaign of November 2013–February 2014 in 60 out of 76 participating children of who parents specifically gave informed consent for HRV measurements. The school is located near to a main road and within a buffer of 1000 m the land cover consists of residential (48.4%) and agricultural (43.1%) areas (Fig. 1). 36 Children were examined three times, 18 children were examined twice, and 6 children were examined once. The repeated measures were on average 46 days apart (range 29–64 days). The clinical visits were scheduled on weekdays between 09.00 AM and 12.00 PM at the school. The children were examined on the same weekday and time point across the clinical visits. The parents were asked to fill out a questionnaire to obtain information on the child's residence, health status, ethnicity, smoking habits of the parents, means of transportation to and from the school and maternal and paternal socio-economic status. Written informed consent from the parents and oral consent from the children was obtained. The study was approved by the Medical Ethics Committee of Hasselt University and the Hospital East-Limburg in Belgium.

#### 2.2. Heart rate variability (HRV) measurements

HRV was measured for 10 min using the Zephyr Biomodule BH3 single-lead ECG monitor mounted on a Zephyr Biopatch (Procare, Groningen, the Netherlands) while the child was seated and had rested for 5 min in the examination room. Standard deviation of normal-to-normal intervals (SDNN), the square root of the mean squared difference of normal-to-normal intervals (rMSSD), high frequency (HF) (0.15 to 0.4 Hz), and low frequency (LF) (0.04 to 0.15 Hz) were computed with a Fast Fourier transformation using the R Heart Rate Variability (RHRV) project (Rodriguez-Linares et al., 2011). Quality control was performed for the 10-min measurement interval of ECG measurements and potential artifacts in the RR measurements were removed manually. The intraclass correlation coefficient of the repeated HRV over different days (on average 46 days apart; range 29–64 days) were 0.21 for SDNN, 0.20 for rMSSD, 0.20 for HF, and 0.27 for LF.

### 2.3. Particulate matter air pollution monitoring

#### 2.3.1. PM measurements at school

A portable laser-operated aerosol mass analyzer (Aerocet 531, Met One Instruments Inc., USA) was used to measure PM. The Aerocet instrument measures  $PM_{2.5}$  (particulate matter with an aerodynamic diameter < 2.5  $\mu m$ ) and  $PM_{10}$  (particulate matter with an aerodynamic diameter < 10  $\mu m$ ) mass concentrations in  $\mu g/m^3$  using a laser-diode-base optical sensor based on light-scattering to measure, distinguish and count the particles. The instrument was calibrated at the factory using NIST traceable polystyrene spheres and compared with the concentrations of an official monitoring station of the Flemish Environment Agency (Borgerhout, Antwerp, Belgium) (Jacobs et al., 2010).

Recent outdoor PM<sub>2.5</sub> and PM<sub>10</sub> was measured at school between 08.00 AM and 12.30 AM and averaged for 3 consecutive sampling periods of 10 min each (measures for 2-min intervals; n = 15). We calculated recent PM<sub>coarse</sub> exposure at school by subtracting PM<sub>10</sub> with PM<sub>2.5</sub> exposure. Indoor PM<sub>10</sub> exposure was measured in the examination room between 08.00 AM–12.30 AM and averaged for 2 consecutive sampling periods of 100 min each (measures for 2-min intervals; n = 100).

#### 2.3.2. PM measurements at residence

The 24-h mean residential exposure to  $PM_{2.5}$  and  $PM_{10}$ was modelled with a high resolution spatial temporal interpolation model. A detailed description can be found elsewhere (Saenen et al., 2016). Daily averages of outdoor temperature and humidity were obtained from the Belgian Royal Meteorological Institute (Brussels, Belgium) and were used to calculate the apparent temperature with the August-Roche-Magnus approximation.

#### 2.4. Relative mitochondrial DNA measures in buccal cells

Buccal cells were collected during each clinical visit using buccal swabs (SK2, Isohelix, Kent, UK) and immediately kept on ice until storage at -80 °C. DNA from buccal cells was extracted using QIAamp DNA micro kit (QIAGEN, N.V. Venlo, the Netherlands). The



Fig. 1. Study area with indication of the school location ( $\blacktriangle$ ), the major roads, annual PM<sub>2.5</sub> exposure based on spatial interpolation models for reference year 2012, and CORINE land cover data based on reference year 2006. Within a buffer of 1000 m, the school is located in 48.4% residential area, 43.1% agricultural, 6.8% natural, and 1.7% industrial area.

concentration and purity of total DNA was measured with Nanodrop spectrophotometer (ND-1000; Isogen Life Science, De Meern, the Netherlands). The average yield  $\pm$  SD of total DNA was 3.6  $\pm$  1.9 µg with  $A_{260}/_{280}$  ratio of 1.93  $\pm$  0.10 and  $A_{260}/_{230}$  ratio of 2.32  $\pm$  0.35. Extracted DNA was stored at -80 °C until further use. Relative mitochondrial DNA content (mtDNAc) was determined by a modified version of a quantitative real-time PCR (qPCR) assay (Janssen et al., 2012; Pieters et al., 2013). Briefly, mitochondrial DNA content was determined by taking the ratio of two mitochondrial gene copy numbers (MTF3212/3319 and MT-ND1) to one reference gene (36B4). Each sample was run in triplicate. A 10 µl PCR reaction mixture contained  $1 \times$  Qiagen Quantitect Sybr Green Mastermix, 2 mM of dithiothreitol, forward (300 nM) and reverse (300 nM) primer and 12.5 ng DNA. All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal cycling profile was similar for mitochondrial DNA and nuclear DNA: 20 s at 95 °C to activate 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. After thermal cycling, raw data were normalized to the nuclear reference gene using qBase software (Biogazelle, Zwijnaarde, Belgium). Cq values of technical replicates were included and averaged when the difference in Cq value was < 0.50.

#### 2.5. Statistics

HRV outcome measurements and mtDNAc were logarithmically  $(\log_{10})$  transformed to improve normality of the distribution. The association between HRV outcome measurements and PM exposure was assessed by performing pollutant exposure response analyses using mixed-effects models that included repeated measurements for each participant (SAS version 9.3, SAS Institute Inc., Cary, NC). We adjusted for the following potential confounders and covariates which were selected a priori: sex, age, body mass index (BMI) and heart rate. The model was additionally adjusted for apparent temperature as an indicator of seasonal variations. In order to account for non-linear effects

of temperature on HRV, we modelled this variable using restricted cubic splines with three knots. To test whether mtDNAc modified the association between HRV outcome measurements and PM exposure, an interaction term between  $\log_{10}$  mtDNAc  $\times$  PM exposure was included. Results show the effect estimates [95% confidence intervals (CI)] of the association between HRV outcome measurements and a 10 µg/m<sup>3</sup> increment in PM exposure modified by mtDNAc over the entire range. Finally, in a sensitivity analysis we additionally adjusted our main models for allergy, asthma or other respiratory-related conditions (based on questionnaire information).

### 3. Results

#### 3.1. Demographic characteristics and PM exposure concentrations

Characteristics of the participating children (n = 60) are given in Table 1. In general, this subgroup did not differ in their characteristics from the COGNAC panel study (Table A.1). There were 39 girls included in the study. The children were approximately 9.9 [1.2] years old and had a body mass index (BMI) of 17.1 [2.9]. We tested whether

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Characteristics of the study population averaged over the examination days.

Characteristics	Mean [SD], median [IQR]* or no. (%)				
Number of subjects	60				
Girls	31 (51.7)				
Age, years	9.9 [1.2]				
Height, cm	141.6 [9.8]				
Weight, kg	34.8 [9.2]				
BMI, kg/m <sup>2</sup>	17.1 [2.9]				
Median SDNN, ms <sup>2</sup>	61.7 [33.6]*				
Median rMSSD, ms <sup>2</sup>	44.1 [42.5]*				
Median HF power	358.3 [671.0]*				
Median LF power	608.2 [623.0]*				
Heart rate, bpm	82.3 [14.0]*				

sex, BMI, passive smoking (exposed-non-exposed), physical activity (hours/week), socio-economic status of either parent (based on highest occupation and highest education of either parent) were associated with HRV measurements. We observed sex-specific differences for all HRV outcomes, which were higher for boys compared with girls (p < 0.05). The other covariates were not associated with HRV measurements.

Median [25th–75th] recent outdoor  $PM_{2.5}$  and  $PM_{10}$  exposure at school was 6.20 [2.8–12.8]  $\mu$ g/m<sup>3</sup> and 29.3 [24.7–42.0]  $\mu$ g/m<sup>3</sup>, respectively.  $PM_{coarse}$  exposure was 24.3 [16.9–28.9]  $\mu$ g/m<sup>3</sup> and indoor  $PM_{10}$  was 26.9 [22.1–30]  $\mu$ g/m<sup>3</sup>. Residential  $PM_{2.5}$  and  $PM_{10}$  exposure were 13.2 [6.9–19.9]  $\mu$ g/m<sup>3</sup> and 14.9 [10.4–25.4]  $\mu$ g/m<sup>3</sup>, respectively.

#### 3.2. Recent PM exposure, relative mtDNAc, and HRV outcome measures

First, we evaluated the association between recent PM exposure and HRV. In mixed-effects models adjusted for sex, age, BMI, heart rate and apparent temperature, we observed a lowering of 3.59% (95% CI: 7.02 to -0.05%, p = 0.05) in the LF parameter for a  $10 \,\mu g/m^3$  increment in outdoor PM<sub>10</sub> exposure. For indoor PM<sub>10</sub> exposure ( $10 \,\mu g/m^3$  increment), we found a lowering in rMSSD (-10.0%; 95% CI: -18.7 to -0.44%, p = 0.04), HF (-19.8%; 95% CI: -32.4 to -4.7%, p = 0.01), and LF (-14.4%; 95% CI: -26.2 to -0.71%, p = 0.04). None of the other exposures were associated with the HRV indicators (data not shown).

Further, we investigated the association between recent PM exposure and relative mtDNAc, and found that a  $10 \,\mu\text{g/m}^3$  increment in outdoor PM<sub>10</sub> or PM<sub>coarse</sub> exposure was associated with a -3.39% (95% CI: -6.07 to -0.64%, p = 0.02) and -5.27% (95% CI: -9.44 to -0.89%, p = 0.01) lowering in mtDNAc, respectively. There was no association of the other exposure indicators with mtDNAc (data not shown).

Finally, we examined the association between mtDNAc and HRV indicators and showed that a 10% increment in mtDNAc was associated with a 1.5% (95% CI: 0.52 to 2.49%, p = 0.003) increase in SDNN, 1.53% (95% CI: 0.06 to 3.02%, p = 0.04) increase in rMSSD, 3.33% (95% CI: 0.82 to 5.90%, p = 0.01) increase in HF, and 2.79% (95% CI: 0.69 to 4.94%, p = 0.01) increase in LF.

## 3.3. Recent PM exposure and HRV outcome measures, dependent on relative mtDNAc

As presented in Figs. 2 and 3, children with low buccal mtDNAc showed a different response to recent outdoor PM exposure than children with high buccal mtDNAc. We found significant air pollution by buccal mtDNAc interaction [log<sub>10</sub>(mtDNAc) × PM exposure] for SDNN ( $p_{int} = 0.03$ ) and LF ( $p_{int} = 0.007$ ) in association with outdoor PM<sub>2.5</sub> exposure and for SDNN ( $p_{int} = 0.02$ ), HF ( $p_{int} = 0.04$ ) and LF ( $p_{int} = 0.005$ ) in association with outdoor PM<sub>10</sub> exposure. For recent PM<sub>coarse</sub> exposure, we observed comparable significant interactions as for outdoor PM<sub>10</sub> exposure: SDNN ( $p_{int} = 0.04$ ), HF ( $p_{int} = 0.04$ ) and LF ( $p_{int} = 0.02$ ) (Fig. A.1). For residential PM<sub>2.5</sub> and PM<sub>10</sub> exposure, we observed a significant interaction term in association with the LF parameter ( $p_{int} = 0.01$  and  $p_{int} = 0.04$ , respectively) (Figs. A.2 and A.3). The interactions for indoor PM<sub>10</sub> exposure were not significant.

Based on the significant interaction terms, the point estimates for the association between HRV indicators and recent outdoor PM exposure are shown in Table 2 for low and high buccal mtDNAc (based on the 25th and 75th percentile of mtDNAc). In children with low buccal mtDNAc, higher PM<sub>2.5</sub> concentrations ( $10 \mu g/m^3$  increment) showed a lowering in the LF parameter with 9.76% (95% CI: -16.9 to -1.99%, p = 0.02), whereas in children with high buccal mtDNAc the association between LF and PM<sub>2.5</sub> exposure was not observed. Each  $10 \mu g/m^3$ increment in PM<sub>10</sub> exposure was inversely associated with three HRV indicators in children with low mtDNAc: -2.24% (95% CI: -4.27 to -0.16%; p = 0.04) for SDNN, -5.67% (95% CI: -10.5 to -0.59%; p=0.03) for HF and -6.64% (95% CI: -10.7 to  $-2.38\%;\,p=0.003)$  for LF.

#### 3.4. Sensitivity analysis

Additional adjustment of the main models of outdoor  $PM_{2.5}$  and  $PM_{10}$  exposure with a variable indicating whether a child had wheezing symptoms (n = 1) or an allergy (n = 14; either against medication use, rhinitis, fruit, pets, house dust, or intolerance to milk) did not alter the main results (Table A.2).

#### 4. Discussion

This study describes for the first time the effect of recent exposure to PM air pollution on HRV in children. We measured rMSSD and HF which reflect parasympathetic cardial vagal tone, SDNN which is an overall measure of changes in autonomic tone and LF variability linked to both sympathetic and parasympathetic nervous system (Laborde et al., 2017). We demonstrated a significant modifying effect of mtDNA content status, as measured in buccal DNA, on the association between recent outdoor PM exposure and HRV in children. Significant reductions in HRV with increasing PM concentrations were only observed in children with a low buccal mtDNA content, which suggests that mtDNAc status may influence the susceptibility to recent PM-linked effects.

MtDNA content, an established marker of mitochondrial damage and dysfunction (Hou et al., 2010; Sahin et al., 2011), is thought to reflect the cumulative burden of oxidative stress, since mitochondria are the most important intracellular source of reactive oxygen species (ROS) and subsequently a target of ROS (Mikhed et al., 2015). Air pollution exposure can lead to increased oxidative stress and mtDNA damage (Grevendonk et al., 2016), with several lines of research suggesting that this damage is linked to age-related endothelial and vascular dysfunction (Mikhed et al., 2015; Forstermann, 2008). External and internal exposure to ROS might determine the mtDNAc in a dynamic way by several feedback mechanisms to guarantee the cellular energy pathways. Studies in adults that link air pollution with blood mitochondrial abundance, as reflected by mitochondrial DNA content, show both increased and decreased associations (Hou et al., 2010; Hou et al., 2013). We found both in the present study in children and in newborns (Janssen et al., 2012; Clemente et al., 2016) lower mtDNAc in association with air pollution. Under the challenge of both endogenous and exogenous ROS production, increased mitochondrial DNA copy number in aging tissues is an acute feedback response that compensates for decreased cellular energy metabolism (Lee and Wei, 2005). The mitochondrial abundance might result in increased oxidative stress levels, which can lead to decreased or no synthesis of mitochondria due to severe oxidative cell damage (Clemente et al., 2017). Therefore, lower mtDNAc might form the biological basis of the inverse association of HRV and recent PM exposure.

Drugs that modify oxidant defenses may influence the susceptibility to particle-induced inflammatory or pro-oxidative responses. Statins have in addition to their cholesterol lowering effect also potent antiinflammatory properties. In this regard, the Normative Aging Study investigators found that the effect of  $PM_{10}$  on heart variability was confined to persons missing the allele for GSTM1 (lower oxidative stress defense), but the association was only apparent in participants without statin treatment (Schwartz et al., 2005). Further effect modification of anti-oxidative properties on the association between air pollution and HRV has been shown by dietary antioxidants and vitamin B, as well as u-3 polyunsaturated fatty acids (Baccarelli et al., 2008; Schwartz et al., 2005; Park et al., 2006). These findings imply that pathways involved in decreasing endogenous oxidative stress have a protective effect which can alleviate reductions in HRV due to exposure of PM air pollution exposure.

Studies on the PM-HRV association have focused only on adults



Fig. 2. Association between HRV indicators and outdoor  $PM_{2.5}$  exposure, with effect modification of mtDNAc. Effect estimates (continuous line) and 95% confidence intervals (grey shading) between heart rate variability (HRV) indicators and outdoor  $PM_{2.5}$  exposure for the range of mtDNAc. The model was adjusted for gender, age, BMI and heart rate. HRV measurements and mtDNAc were log transformed.  $P_{int}$  is based on the continuous association of  $log_{10}(mtDNAc) \times PM_{2.5}$  exposure model.

(Pieters et al., 2012), and suggest that reduced HRV is one of the mechanistic routes that lead to PM-induced negative health effects (Park et al., 2005; Pope III et al., 2004). We investigated the possible effects of acute PM exposure in children because findings in adults may not be generalized to children because of potential different exposure patterns and age-dependent differences in autonomic functioning. We postulate that children may be more susceptible to the adverse effects of air pollution due to their relatively higher ventilation rate and metabolic turnover, and the fact that organ systems including the immune system are still in development (Kim, 2004). In addition, children tend to be more physically active and spend more time outdoors, which could contribute to their increased vulnerability towards effects of airborne particles (Kim, 2004).

We used buccal mtDNA content as *a proxy* for oxidative stress and found that high mtDNA content was protective for PM-induced HRV changes. In this respect, it was recently shown that blood mtDNA content caused effect modification in the negative association between ambient black carbon levels and blood pressure in older men, with high mtDNA content attenuating the effect (Zhong et al., 2016). In the present study, we found for outdoor PM<sub>10</sub> significant mtDNAc × PM interactions for SDNN, HF and LF while for outdoor PM<sub>2.5</sub> exposure only one significant mtDNAc × PM interaction was observed. The PM<sub>coarse</sub> fraction showed comparable interactions as for outdoor PM<sub>10</sub>. These results indicate that some of the associations are induced by the larger particle fraction. Brunekreef and Forsberg (2005) have systematically evaluated the existing data on health effects of the coarse fraction  $(PM_{2.5-10})$  and  $PM_{2.5}$ , and concluded that there is evidence to suggest that both these PM fractions are able to elicit adverse health effects.

The present study has some strengths and limitations. Our findings are based on exposure measurements at school. HRV measurements were performed in standardized conditions. We used repeated examinations of heart rate variability within the same child. Withinsubject repeated measures allow each subject to act as its own control, which reduces potential bias due to unmeasured confounding factors. A first limitation is that the HRV measures in standardized conditions only represents the HRV indexes at rest and are not based on a longterm e.g. 24 h assessment. Second, because of ethical difficulties in obtaining blood from children, we used buccal cells as biological sample to assess mtDNAc. Therefore, we were not able to compare the mtDNAc of buccal cells with other biological matrices such as blood. A limitation is that mtDNAc may vary among different biological samples, so interpretation of our results may be limited as mtDNAc in buccal cells may not be representative for the human body. Furthermore, buccal swabs represent a heterogeneous mixture of buccal epithelial cells and leukocytes, which we could not account for in this study.



**Fig. 3.** Association between HRV indicators and outdoor  $PM_{10}$  exposure, with effect modification of mtDNAc. Effect estimates (continuous line) and 95% confidence intervals (grey shading) between HRV indicators and outdoor  $PM_{10}$  exposure for the range of mtDNAc. The model was adjusted for gender, age, BMI and heart rate. HRV measurements and mtDNAc were log transformed.  $P_{int}$  is based on the continuous association of  $log_{10}$ (mtDNAc) ×  $PM_{10}$  exposure model.

#### Table 2

Change in HRV measurements (%) in association with recent outdoor PM exposure and effect-modification by mtDNAc.

HRV component	mtDNAc	% change	95% CI	p-Value	$p_{int}$ (mtDNAc $\times$ PM exposure)		
Effect of PM <sub>10</sub> on HRV, by mtDNAc							
SDNN	25th percentile	-2.24	-4.27 to -0.16	0.04	0.02		
	75th percentile	0.25	-1.56 to 2.10	0.79			
rMSSD	25th percentile	-2.74	-5.71 to 0.30	0.08	0.06		
	75th percentile	0.65	-2.29 to 3.69	0.66			
HF	25th percentile	-5.67	-10.5 to -0.59	0.03	0.04		
	75th percentile	0.84	-4.08 to 6.01	0.74			
LF	25th percentile	-6.64	-10.7 to -2.38	0.003	0.005		
	75th percentile	-0.78	-3.52 to 5.28	0.72			
Effect of PM <sub>25</sub> on HRV, by mtDNAc							
SDNN	25th percentile	-3.01	-6.62 to 0.74	0.11	0.03		
	75th percentile	1.74	-1.89 to 5.50	0.34			
rMSSD	25th percentile	-3.65	-9.11 to 2.15	0.21	0.12		
	75th percentile	1.48	-4.01 to 7.29	0.60			
HF	25th percentile	-6.01	-14.8 to 3.70	0.21	0.12		
	75th percentile	2.97	-6.23 to 13.1	0.53			
LF	25th percentile	-9.76	-16.9 to -1.99	0.02	0.007		
	75th percentile	2.84	-5.08 to 11.43	0.49			

Percent change (95% CI) in HRV for an increment of  $10 \,\mu\text{g/m}^3$  in recent outdoor PM<sub>10</sub> or PM<sub>2.5</sub> exposure, adjusted for gender, age, BMI and heart rate. Predicted estimates are presented for mtDNAc status at the 25th (-1.10) and 75th percentile (1.48) of the distribution. HRV measurements and mtDNAc were log<sub>10</sub> transformed. p-Value represents the significance of the HRV-PM association, modified for mtDNAc.

#### 5. Conclusion

Our findings, in accordance with observation in adults (Pieters et al., 2012), support the hypothesis that exposure to PM air pollution leads to rapid changes in the autonomic nervous regulation of children. Indeed, in children with low mitochondrial DNA content, air pollution might decrease the sympathetic predominance of HRV. Other lifestyle determinants acting via inflammatory or oxidative pathways and the modification by mitochondrial function must still be elucidated. Our findings might open new perspectives for risk stratification and individualized cardiovascular prevention early in life.

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#### Appendix A

#### Table A.1

General characteristics of the three schools participating in the COGNAC panel study.

	School 1	School 2	School 3 (HRV)		
Age, years	$10.33 \pm 0.98$	$10.54 \pm 1.33$	$9.9 \pm 1.2$		
Length, cm	144.9 ± 9.2	146.0 $\pm 10.4$	141.6 ± 9.8		
BMI	$36.9 \pm 10.2$	$37.6 \pm 9.61$	$34.8 \pm 9.2$		
	17.4 ± 3.38	17.4 ± 2.70	17.1 ± 2.9		
Girl	49.80%	48.70%	51.70%		

#### Table A.2 Sensitivity analysis.

Main model	HRV component	mtDNAc	PM <sub>10</sub> exposure				PM <sub>2.5</sub> exposure					
			% change	95% CI			p-Value	% change	95% CI			p-Value
+allergy/asthma child	SDNN	25th percentile	-2.25	-4.28	to	-0.17	0.03	-2.78	-6.43	to	1.02	0.15
		75th percentile	0.73	-1.29	to	2.79	0.47	1.97	-1.69	to	5.77	0.29
	rMSSD	25th percentile	-2.50	-5.56	to	0.65	0.12	-3.34	-8.90	to	2.55	0.25
		75th percentile	0.95	-2.11	to	4.10	0.54	1.79	-3.79	to	7.69	0.53
	HF	25th percentile	-5.40	-10.4	to	-0.10	0.05	-5.58	-14.6	to	4.40	0.26
		75th percentile	1.26	-3.91	to	6.71	0.63	3.57	-5.90	to	14.0	0.47
	LF	25th percentile	-6.44	-10.7	to	-2.03	0.005	-9.56	-16.9	to	-1.63	0.02
		75th percentile	1.06	-3.41	to	5.75	0.64	3.11	- 4.96	to	11.9	0.45

Percent change (95% CI) in HRV for an increment of  $10 \,\mu\text{g/m}^3$  in PM<sub>10</sub> or PM<sub>2.5</sub> exposure. p-Value represents the significance of the PM-HRV association, modified for mtDNAc.

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#### **Competing interests**

directions for participating in this study.

The authors declare they have no conflict of interest.



Fig. A.1. Association between HRV indicators and  $PM_{coarse}$  exposure, with effect modification of mtDNAc. Effect estimates (continuous line) and 95% confidence intervals (grey shading) between heart rate variability (HRV) indicators and  $PM_{coarse}$  exposure for the range of mtDNAc. The models were adjusted for gender, age, BMI and heart rate. HRV measurements and mtDNAc were log transformed.



Fig. A.2. Association between HRV indicators and residential  $PM_{2.5}$  exposure, with effect modification of mtDNAc. Effect estimates (continuous line) and 95% confidence intervals (grey shading) between heart rate variability (HRV) indicators and residential  $PM_{2.5}$  exposure for the range of mtDNAc. The model was adjusted for gender, age, BMI and heart rate. HRV measurements and mtDNAc were log transformed.



**Fig. A.3.** Association between HRV indicators and residential  $PM_{10}$  exposure, with effect modification of mtDNAc. Effect estimates (continuous line) and 95% confidence intervals (grey shading) between heart rate variability (HRV) indicators and residential  $PM_{10}$  exposure for the range of mtDNAc. The model was adjusted for gender, age, BMI and heart rate. HRV measurements and mtDNAc were log transformed.

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