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Child buccal telomere length and mitochondrial DNA content as biomolecular markers of ageing in association with air pollution

Pauline Hautekiet [a,b], Tim S. Nawrot [a,c,*], Bram G. Janssen [a], Dries S. Martens [a], Eva M. De Clercq [b], Payam Dadvand [d,e,f], Michelle Plusquin [a], Esmée M. Bijnens [a], Nelly D. Saenen [a,b]

[a] Centre for Environmental Sciences, Hasselt University, Agoralaan gebouw D, BE-3590 Hasselt, Belgium
[b] Risk and Health Impact Assessment, Sciensano, Juliette Wytsmanstraat 14, BE-1050 Brussels, Belgium
[c] Department of Public Health & Primary Care, University of Leuven (KU Leuven), O&N I Herestraat 49 - bus 706, BE-3000 Leuven, Belgium
[d] ISGlobal, Campus Mar, Dr Aiguader 88, ES-08003 Barcelona, Spain
[e] Pompeu Fabra University, Doctor Aiguader 80, 08003 Barcelona, Catalonia, Spain
[f] Ciber on Epidemiology and Public Health (CIBERESP), Melchor Fernández Almagro 3-5, 28029 Madrid, Spain

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ABSTRACT

Background: Pro-inflammatory conditions such as air pollution might induce biological ageing. However, the available evidence on such an impact in children is still very scarce. We studied in primary schoolchildren the association of ambient residential air pollution exposure with telomere length (TL) and mitochondrial DNA content (mtDNAc), two important targets of the core axis of ageing.

Methods: Between 2012 and 2014, buccal TL and mtDNAc were repeatedly assessed using qPCR in 197 Belgian primary schoolchildren (mean age 10.3 years) as part of the COGNAC study. At the child's residence, recent (week), sub-chronic (month) and chronic (year) exposure to nitrogen dioxide (NO_2), particulate matter ≤ 2.5 μm ($PM_{2.5}$) and black carbon (BC) were estimated using a high resolution spatiotemporal model. A mixed-effects model with school and subject as random effect was used while adjusting for *a priori* chosen covariates.

Results: An interquartile range (IQR) increment (1.9 μg/m^3) in chronic $PM_{2.5}$ exposure was associated with a 8.9% (95% CI: −15.4 to −1.9%) shorter TL. In contrast to $PM_{2.5}$, chronic exposure to BC and NO_2 was not associated with TL but recent exposure to BC and NO_2 showed significant inverse associations with TL: an IQR increment in recent exposure to BC (0.9 μg/m^3) and NO_2 (10.2 μg/m^3) was associated with a 6.2% (95% CI: −10.6 to −1.6%) and 6.4% (95% CI: −11.8 to −0.7%) shorter TL, respectively. Finally, an IQR increment in chronic $PM_{2.5}$ exposure was associated with a 12.7% (95% CI: −21.7 to −2.6%) lower mtDNAc. However, no significant associations were seen for NO_2 and BC or for other exposure windows.

Conclusion: Chronic exposure to $PM_{2.5}$ below the EU threshold was associated with child's shorter buccal TL and lower mtDNAc, while traffic-related pollutants (BC and NO_2) showed recent effects on telomere biology. Our data add to the literature on air pollution-induced effects of TL and mtDNAc, two measures part of the core axis of cellular ageing, from early life onwards.

1. Introduction

Telomeres are the end caps of chromosomes and consist of multiple TTAGGG sequence repeats (Blackburn 1991). Their main function is to protect chromosomes from degradation. Telomeres shorten with every cell division because of the 'end-replication problem' and are therefore considered as a biomarker of ageing (Levy et al. 1992). This is confirmed by studies in adults showing the association between telomere length (TL) and several age-related diseases like atherosclerosis (Ormseth et al. 2016), Alzheimer's disease (Zhan et al. 2015) and type 2 diabetes mellitus (Willeit et al. 2014). Besides adults, TL has also been assessed in children. Research showed that TL in placental tissue was positively

Abbreviations: $PM_{2.5}$, particulate matter with a diameter ≤ 2.5 μm; BC, black carbon; NO_2, nitrogen dioxide; TL, telomere length; mtDNAc, mitochondrial DNA content; IQR, interquartile range; Cq, cycle quantification value.

* Corresponding author at: Hasselt University, Centre for Environmental Sciences, Agoralaan gebouw D, BE-3590 Hasselt, Belgium.
E-mail address: tim.nawrot@uhasselt.be (T.S. Nawrot).

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associated with buccal TL in young adulthood, emphasizing the importance of determining early-life TL (Bijnens et al. 2017). Furthermore, early life TL has been shown to predict the lifespan of zebra finches (Heidinger et al. 2012).

Telomere attrition is a dynamic process throughout the life course (Martens and Nawrot 2016) and cellular division is not the only mechanism that affects it. On a cellular level, telomeres are believed to be affected by oxidative stress and inflammation due to personal characteristics and lifestyle factors such as obesity (Lamprokostopoulou et al., 2019; Martens et al., 2016) and smoking (Astuti et al. 2017).

A second cell structure that is also targeted by oxidative stress and inflammation is the mitochondrion and together with telomeres they are part of the core axis of ageing (Sahin and DePinho 2012). Mitochondria are crucial to the cell as they are responsible for the cell's energy production (Taanman 1999). Mitochondrial DNA is sensitive to oxidative stress because it lacks the protective histones that are found in nuclear DNA and because it has a less efficient repair mechanism (Lee and Wei 2000). Mitochondrial DNA content (mtDNAc) has a curvilinear association with age (Knez et al. 2016). Similar as for TL, mtDNAc in adults has been associated with age-related diseases like Parkinson's disease (Pyle et al. 2016) and coronary heart disease (Ashar et al. 2017). In children or newborns, mtDNAc has been associated with birth weight (Clemente et al. 2017) as well with intelligence (Bijnens et al. 2019) and it was shown to be an effect modifier between air pollution and heart rate variability (Saenen et al. 2019).

As an important source of oxidative stress and inflammation, air pollution has been identified to play a role in the ageing process (Janssen et al., 2012; Martens and Nawrot, 2016; Martens et al., 2017). Although most studies focused on adults, it is especially important to evaluate this effect in children because (1) early life TL is predictive of longevity as shown in zebra finches (Heidinger et al. 2012), (2) the most rapid decline in TL occurs during childhood (Bijnens et al. 2017), and (3) most of the variation in TL between individuals is determined early in life (Benetos et al. 2019).

In this study, we examined whether ambient residential exposure to nitrogen dioxide (NO_2) and black carbon (BC), two traffic-related air pollutants and particulate matter $\leq 2.5\,\mu m$ ($PM_{2.5}$), a complex multi-pollutant mixture of solid and liquid particles, is associated with TL and mtDNAc in children between 9 and 12 years old. Our hypothesis was that children exposed to higher levels of ambient air pollution had a shorter buccal TL and lower mtDNAc, indicating an accelerated molecular ageing process.

2. Methods

2.1. Study population

This study was part of the COGNAC (COGNition and Air pollution in Children) study, executed between January 2012 and February 2014. In total, we repeatedly invited 770 children between 9 and 12 years old from three different schools, each in another municipality (Tienen, Zonhoven, Hasselt) in Belgium. Parents of the participants signed an informed consent and completed a questionnaire to provide information on the lifestyle of the child and its family, the socioeconomic status of the family and the smoking behavior of the family members. Socioeconomic status was based on the highest educational level of either parents (up to high school diploma – college or university diploma). Children were considered as exposed to passive smoking if one or more family members smoked inside the house. Daily average apparent temperature was obtained from the Royal Meteorological Institute of Belgium. The study followed the Helsinki declaration and was approved by the ethics committees of Hasselt University and the Eastern-Limburg Hospital, Belgium.

Out of 770 children, 334 (43%) agreed to participate. For accurate telomere and mtDNAc measurements a qualitative buccal DNA sample is essential. Also, we used a threshold Cq value (cycle quantification value) of 0.3 as absolute difference in triplicate measurements. Based on these criteria we were able to include qualitative data from 197 children (59%).

2.2. Sample collection

Buccal cell swabs (SK2, Isohelix, Kent, UK) were sampled at the school during school time. Per examination we collected two buccal samples per child. This was repeated three times making sure that each of the three examinations was done at the same time and on the same day of the week. The mean (SD) period of time between two consecutive examinations was 46 (20) days. Of the 197 children included, 91 (46.2%) provided buccal swabs at one time point, 71 (36.0%) at two time points and 35 (17.8%) at three time points, amounting to a total number of 338 observations.

Prior to sampling, the children did not eat or drink for at least 30 min and rinsed their mouth three times with water. Each swab was rubbed inside the cheek for one minute. Afterwards the samples were kept on ice until storage at −80 °C.

2.3. Mitochondrial DNA and telomere length assay

DNA was extracted from the two buccal swabs using the QIAgen Micro Kit (Qiagen, N.V.V Venlo, The Netherlands). The quantity and purity of the sample was measured with a Nanodrop spectrophotometer (ND-1000; Isogen Life Science, De Meern, the Netherlands). To ensure a uniform DNA input for each qPCR reaction, samples were diluted and checked using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Europe). Extracted DNA was stored at −80 °C until analysis.

Average relative TL was measured in buccal cells by determining the ratio of one telomere gene copy number (T) to one reference gene (36B4) (S). Average mtDNAc was measured by determining the average of the ratio of two mitochondrial gene copy numbers (MTF3212/R3319 and MT-ND1) (M) to one reference gene (36B4) (S). Samples were measured in triplicate (3 technical replicates to control for variation) using a previously described quantitative real-time PCR (qPCR) assay with minor modifications (Janssen et al., 2012; Martens et al., 2016). The mitochondrial DNA and reference gene reaction mixture contained Qiagen 2x QuantiTect SYBR Green Mastermix, forward (300 nM) and reverse (300 nM) primer and 12.5 ng DNA. TL reaction mixture contained Qiagen 2x QuantiTect SYBR Green Mastermix, 2 mM of dithiothreitol, telg (300 nM) and telc (900 nM) primer (Cawthon 2009) and 12.5 ng DNA. All PCR-reactions were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following thermal cycling profile: (i) mtDNAc – 1 cycle of 10 min at 95 °C to activate the HotStarTaq® DNA-polymerase, followed by 30 cycles of 15 s at 95 °C for denaturation and 1 min 10 s at 58 °C for annealing/extension; (ii) reference gene – 1 cycle of 10 min at 95 °C, followed by 35 cycles of 15 s at 95 °C and 1 min 10 s at 58 °C; (iii) TL – 1 cycle of 10 min at 95 °C, followed by 2 cycles of 15 s at 94 °C and 2 min at 49 °C, and finally 30 cycles of 15 s at 94 °C, 20 s at 62 °C and 1 min 40 s at 74 °C.

Reaction efficiency was assessed on each plate by using a 6-point serial dilution of DNA. Six inter run calibrators (IRCs) were used to account for inter-run variability. Also, non-template controls were used in each run. Raw data were processed and normalized to the reference gene using qBase plus software (Biogazelle, Zwijnaarde, Belgium), taking into account run-to-run differences.

The reliability of our assay was assessed by calculating the interclass coefficient (ICC) with 95% CI of the triplicate measures (T/S ratios, T and S measures separately) for telomere runs and (M/S ratios, M and S measures separately) for mtDNAc runs using the SPSS statistical package version 25 (IBM Corp., Armonk, NY, USA) based on a mean rating, absolute-agreement, 2-way mixed-effects model. For telomere length, the ICCs (95% CI) of T/S ratios, telomere runs and single-copy runs

were respectively 0.976 (0.971 to 0.981), 0.995 (0.984 to 0.996) and 0.987 (0.985 to 0.990). For mtDNAc, the ICCs (95% CI) of M/S ratios (MTF3212/3319 and MT-ND1), mtDNAc runs (MTF3212/3319 and MT-ND1) and single-copy gene runs were respectively 0.981 (0.976 to 0.984), 0.980 (0.975 to 0.984), 0.989 (0.986 to 0.991), 0.983 (0.979 to 0.986) and 0.987 (0.985 to 0.990). Based on the six inter-run calibrators, the inter-assay ICC was 0.992 (0.988 to 0.995) for telomeres and 0.991 (0.987 to 0.994) and 0.988 (0.982 to 0.992) for mtDNAc (MTF3212/3319 and MT-ND1 respectively).

2.4. Air pollution exposure

Residential addresses of the children were geocoded. Daily residential exposure ($\mu g/m^3$) to $PM_{2.5}$, BC and NO_2 at the child's residence was modelled using a spatiotemporal interpolation model, designed for Belgium (Janssen et al. 2008). This model included land cover data obtained by satellites (CORINE land-cover data set) and pollution data from the Belgian fixed monitoring stations in combination with a dispersion model (Lefebvre et al. 2013; Maiheu et al. 2013). The dispersion model described by Lefebvre et al. (2011, 2013) uses the results from the interpolation method as background and superimposes the effects of industrial point sources and line sources from traffic to calculate the daily concentrations at a high resolution.

The overall model performance was evaluated by leave-one-out cross-validation and was based on 34 monitoring points for $PM_{2.5}$, 44 for NO_2 and 14 for BC. The temporal and spatial variability of the model was explained by 80% for $PM_{2.5}$ (Maiheu et al. 2013), 78% for NO_2 (Maiheu et al. 2013) and 74% for BC (Lefebvre et al. 2011). During a previous study, we validated the modelled exposure by measuring the internal carbon as urinary carbon particles, showing that the internal carbon exposure is correlated with the chronic residential carbon exposure (*partial* $r = 0.17$, CI: 0.05 to 0.28) (Saenen et al. 2017).

We used the daily modelled exposure values obtained by this model to assign the residential exposure during the week (recent), month (sub-chronic) and year (chronic) before each examination. For children with more than one residential address at the moment of the study, we calculated a weighted average using the proportion of time spent at each location.

2.5. Statistical analysis

Statistical analyses were performed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA). We performed a log(10) transformation of the TL and mtDNAc data to reduce skewness and to better approximate a normal distribution.

Given the repeated measurements of our outcomes, we developed mixed-effects models (unstructured covariance matrix) with school and subject as random effect to assess the associations between exposure to air pollution and either TL or mtDNAc (one at a time). *A priori* selected covariates included age (continuous), sex (boy – girl), BMI (continuous), passive smoking (yes – no), season of examination (winter – summer – autumn – spring), highest educational level (low - high) of both parents as an indicator of socioeconomic status and daily average apparent temperature. We chose these covariates as sex, age, BMI, passive smoking and socio-economic status have been associated with the outcomes (Lapham et al., 2015; Lu et al., 2017; Robertson et al., 2013; Skuratovskaia et al., 2019) whereas season and apparent temperature have been associated with the exposures (Cichowicz et al., 2017; Duan et al., 2019).

In order to account for non-linear effects of apparent temperature, we modelled this variable using restricted cubic splines with three knots. All covariates were measured at baseline except apparent temperature and season, which were accounted for at each examination. Finally, we also added a fixed covariate term for examination (1 – 2 – 3). In a second model, we additionally adjusted the main chronic model for recent exposure and the recent and sub-chronic model for chronic exposure. Results were presented as the percentage difference in TL or mtDNAc associated with an interquartile range (IQR) increment in air pollution exposure.

Associations between TL and mtDNAc and between the biomarkers and the fixed covariates in the bivariate analyses were assessed using the unadjusted model, but taking into account the random effect of the subjects. Unadjusted Spearman's correlations were used to assess the associations between the air pollutants at the different time windows.

In a sensitivity analysis, we additionally adjusted the main models of chronic $PM_{2.5}$ and recent BC and NO_2 for ethnicity (both parents born in Belgium – one or both parents born abroad), children's means of transportation to school (by foot – bike – car), neighborhood household income (continuous) or physical activity (hours/week) like for example soccer, basketball, gymnastics, swimming or horseback riding. We chose these potential covariates for a sensitivity analysis as telomere length might be associated with ethnicity (Ly et al. 2019) and physical activity (Tucker 2017) and air pollution might be associated with physical activity (An et al. 2018) and transport mode (Cepeda et al. 2017). To calculate the neighborhood household income, children were assigned to a statistical sector (average area = 1.55 km^2), based on their home address. This is the smallest administrative entity for which statistical data are produced by the Belgian National Institute of Statistics (NIS). Belgian census data derived from the NIS were used to define neighborhood socio-economic status based on annual household income in the year 2012. Finally, in the main models of chronic $PM_{2.5}$ and recent BC and NO_2 we added an interaction term for sex with the predictor to the model and stratified per sex to evaluate effect modification.

3. Results

3.1. Population and exposure characteristics

The characteristics of the 197 children included in this study and the 334 children of the original population are presented in Table 1. The study population is representative of the original population but we notice that relatively more children from the school in Zonhoven and less from the school in Kiewit were included. Of all children, 51.3% were boys and 48.7% were girls. The average age, BMI, weight and length (SD) were respectively 10.3 (1.3) years, 17.5 (3.0) kg m^{-2}, 37.1 (9.6) kg and 144.6 (9.8) cm. The highest education of both parents was for 31.0% up to high school and for 69.0% college or university. 17.3% of the

Table 1
Characteristics of the study population (n = 197) and the original population (n = 334).

Anthropometric characteristics	Mean (SD) or n (%) Study population (n = 197)	Mean (SD) or n (%) Original population (n = 334)
Boys	101 (51.3%)	165 (49.4%)
Age, years	10.3 (1.3)	10.2 (1.3)
Body Mass Index (BMI)	17.5 (3.0)	17.3 (2.9)
Weight, kg	37.1 (9.6)	36.8 (9.5)
Length, cm	144.6 (9.8)	144.8 (10.0)
Lifestyle characteristics		
Highest category of education of either parents[a]		
Up to high school diploma	61 (31.0%)	99 (29.8%)
College or university diploma	136 (69.0%)	233 (70.2%)
Exposed to passive tobacco smoke[b]	34 (17.3%)	53 (15.9%)
School		
Tienen	44 (22.4%)	70 (21.0%)
Zonhoven	83 (42.1%)	188 (56.3%)
Kiewit	70 (35.5%)	76 (22.7%)

[a] Original population: data available for 331 subjects.
[b] Original population: data available for 333 subjects

children were exposed to passive smoking. Average relative TL ranged from 0.50 to 1.98. For boys and girls TL ranged respectively from 0.50 to 1.98 and from 0.54 to 1.92. Average relative mtDNAc ranged from 0.35 to 3.62. For boys and girls mtDNAc ranged respectively from 0.46 to 3.62 and from 0.35 to 2.96.

Bivariate analyses between the ageing markers and study characteristics, adjusted for the random effect of subject, are presented in supplementary table 1. Firstly, 1 unit higher BMI was associated with a 1.5% (95% CI: −2.7 to −0.2, $p = 0.023$) shorter buccal TL. No association was found between buccal TL and age ($p = 0.28$) or sex ($p = 0.77$). Secondly, a one-year increment in age was associated with a 5.4% (95% CI: 1.1 to 9.8, $p = 0.01$) higher buccal mtDNAc. Girls had a 12.9% (95% CI: −22.4 to −3.6, $p = 0.0080$) lower buccal mtDNAc compared with boys and children exposed to passive smoking tended to have a 12.3% (95% CI: −24.1 to 0.7, $p = 0.063$) lower mtDNAc. The adjusted estimated effects of each of the fixed covariates in the main model of chronic $PM_{2.5}$ and TL or mtDNAc are presented in supplementary table 2.

As shown in Supplementary Fig. 1, children's TL at the three examinations were correlated (examination 1–2: r = 0.31, $p = 0.0046$, examination 1–3: r = 0.62, $p < 0.0001$, examination 2–3: r = 0.53, $p < 0.0001$). Similar results were found for mtDNAc (examination 1–2: r = 0.50, $p < 0.0001$, examination 1–3: r = 0.58, $p < 0.0001$, examination 2–3: r = 0.46, $p = 0.0009$) (Suppl. Fig. 2). No association was found between TL and mtDNAc ($p = 0.73$). Mean chronic exposure to $PM_{2.5}$ (IQR) was 14.6 (1.9) μg/m^3. Mean recent exposure to BC and NO_2 (IQR) was respectively 1.6 (0.9) μg/m^3 and 24.1 (10.2) μg/m^3 (Table 2). The different air pollutants and time windows were highly correlated. The highest correlation was found between sub-chronic BC and NO_2 exposure (Spearman's correlation = 0.95, $p < 0.0001$) (Suppl. table 3).

3.2. Air pollution in association with telomere length and mitochondrial DNA content

In the mixed-effects model accounting for the aforementioned covariates, chronic $PM_{2.5}$ exposure was associated with shorter buccal TL. An IQR increment (1.9 μg/m^3) in chronic $PM_{2.5}$ was associated with a 8.9% (95% CI: −15.4 to −1.9%, $p = 0.014$) shorter TL (Fig. 1A). In contrast to $PM_{2.5}$, chronic exposure to BC and NO_2 was not associated with TL but recent exposure to BC and NO_2 showed significant inverse associations with TL. An IQR increment in recent exposure to BC (0.9 μg/m^3) and NO_2 (10.2 μg/m^3) was respectively associated with a 6.2% (95% CI: −10.6 to −1.6%, $p = 0.0091$) and 6.4% (95% CI: −11.8 to −0.7%, $p = 0.029$) shorter TL. Additionally adjusting the recent and sub-chronic exposure model for chronic exposure and the chronic exposure model for recent exposure did not result in a notable change of the associations of the main analysis (Fig. 1B).

Secondly, a significant association was observed between chronic $PM_{2.5}$ exposure and mtDNAc. An IQR increment in $PM_{2.5}$ (1.9 μg/m^3) was associated with a 12.7% (95% CI: −21.7 to −2.6%, $p = 0.016$) lower mtDNAc (Fig. 2A). We did not observe any association between recent or sub-chronic exposure to $PM_{2.5}$, BC, or NO_2 and mtDNAc. Detailed estimates of the main analyses are shown in Supplementary table 4. When we additionally adjusted the recent and sub-chronic model for chronic exposure and the chronic model for recent exposure, similar associations as the main analysis were found (Fig. 2B).

Table 2
Ambient residential exposure characteristics.

	Mean	25th percentile	50th percentile	75th percentile
Recent exposure: week (μg/m^3)				
$PM_{2.5}$	16.6	8.1	13.3	21.5
BC	1.6	1.1	1.4	1.9
NO_2	24.1	18.8	23.6	29.0
Sub-chronic exposure: month (μg/m^3)				
$PM_{2.5}$	13.6	8.4	10.5	17.6
BC	1.4	1.1	1.3	1.7
NO_2	21.5	17.6	20.5	24.2
Chronic exposure: year (μg/m^3)				
$PM_{2.5}$	14.6	13.7	14.7	15.6
BC	1.5	1.3	1.5	1.6
NO_2	20.7	19.4	20.7	22.0

3.3. Sensitivity analysis

The characteristics of the variables used in the sensitivity analysis are presented in Supplementary table 5. In a sensitivity analysis, additional adjustment of our analyses for ethnicity or neighborhood household income did not result in a notable change of the aforementioned associations observed in our main analyses (Table 3). Additional adjustment for physical activity in the association between recent NO_2 and TL resulted in a reduced significance whereas it gained significance in the association with mtDNAc. Additional adjustment for children's means of transportation to school in the association between chronic $PM_{2.5}$ and TL changed to a marginally significant result. After stratification for sex, we observed significant associations between TL and recent NO_2, recent BC and chronic $PM_{2.5}$ for boys but not for girls (Suppl. table 6). For example, an IQR increment in chronic $PM_{2.5}$ was associated with a 13.0% (95% CI: −21.4 to −3.6%, $p = 0.0082$) lower TL in boys whereas no association was found for girls ($p = 0.46$). On the other hand, an IQR increment in chronic $PM_{2.5}$ was associated with a 18.1% (95% CI: −31.1 to −2.7%, $p = 0.024$) lower mtDNAc for girls whereas this association was not found for boys ($p = 0.27$).

4. Discussion

Telomere length and mitochondrial DNA are part of the core axis of ageing (Martens and Nawrot, 2016; Sahin and DePinho, 2012). In 9 to 12 years old children, we investigated whether ambient air pollution is associated with these biomarkers. We found that higher exposure to ambient residential air pollution was associated with a shorter buccal TL and a lower mtDNAc in children. Additionally adjusting the recent and sub-chronic exposure model for chronic exposure and the chronic exposure model for recent exposure showed similar results as the main analysis.

Firstly, we evaluated the effects of air pollution on TL. Telomere length is a marker of biological ageing that may provide a cellular memory of exposures to oxidative stress and inflammation (Martens and Nawrot 2016). Early-life TL has been related to life expectancy in zebra finches (Heidinger et al. 2012) and might be the most important determinant of TL in adulthood (Bijnens et al. 2017). Our results showed an inverse association between air pollution exposure and buccal TL in children. Two recent systematic reviews and meta-analyses of the available evidence support this inverse association (Miri et al., 2019; Zhao et al., 2018). Telomere length was associated with recent exposure to the traffic-related air pollutants (BC and NO_2) and with chronic exposure to $PM_{2.5}$. This suggests a difference between traffic and non-traffic related air pollutants. Particulate matter is known to have several sources and mass compositions which may indicate differences in toxicity (De Kok et al. 2006). Therefore, recent PM sources might differ from chronic sources. Also, as traffic only represents part of the PM exposure, the effects might differ from the traffic-related pollutants (De Kok et al., 2006; Kim et al., 2015; Thurston et al., 2016). The difference in effect between traffic and non-traffic related air pollution should be evaluated in future studies.

Until now, only four studies reported on the association between exposure to air pollution and TL in children, of which three studies found similar results (Clemente et al., 2019; Lee et al., 2017; Moslem et al., 2020) and one study showed contrasting results (Walton et al. 2016). The cross-sectional study by Lee et al. (2017) included 14

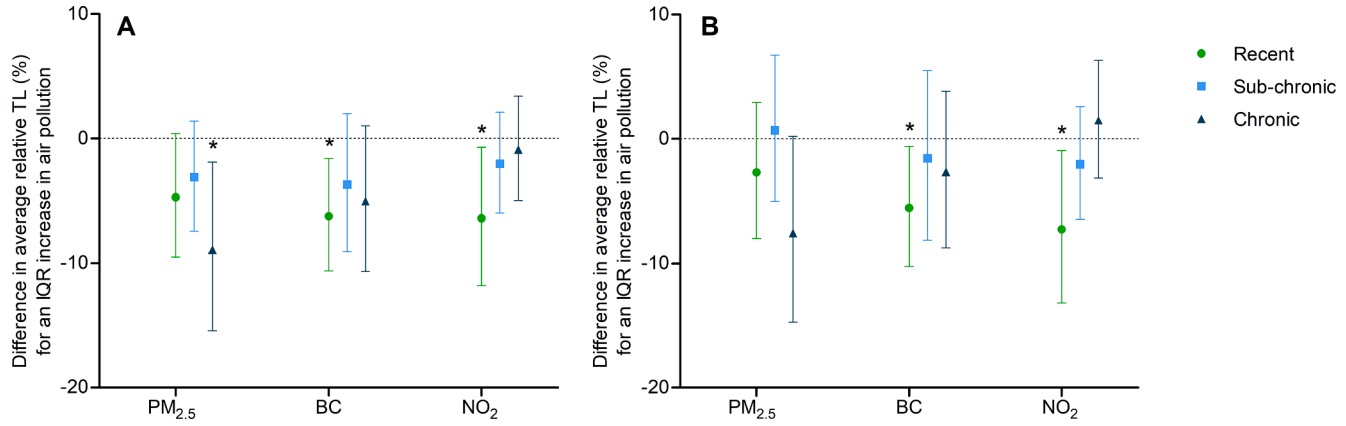

Fig. 1. Difference (%) in average relative telomere length (TL) (with 95% CI) in association with recent (week), sub-chronic (month) and chronic (year) exposure to $PM_{2.5}$, NO_2 and BC. Figure A shows the results of the main analysis. Figure B shows the results after additionally adjusting the chronic model for recent exposure and the recent and sub-chronic model for chronic exposure. Estimates were adjusted for sex, age, BMI, socioeconomic status, passive smoking, season of examination, examination, apparent temperature and the random effect of school and subject. $^*p < 0.05$.

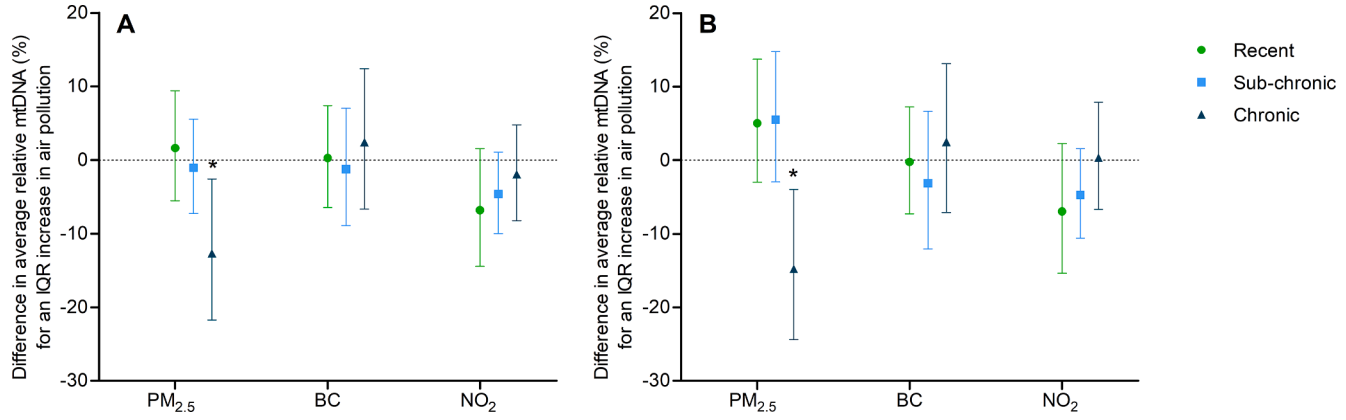

Fig. 2. Difference (%) in average relative mitochondrial DNA content (mtDNAc) (with 95% CI) in association with recent (week), sub-chronic (month) and chronic (year) exposure to $PM_{2.5}$, NO_2 and BC. Figure A shows the results of the main analysis. Figure B shows the results after additionally adjusting the chronic model for recent exposure and the recent and sub-chronic model for chronic exposure. Estimates were adjusted for sex, age, BMI, socioeconomic status, passive smoking, season of examination, examination, apparent temperature and the random effect of school and subject. $^*p < 0.05$.

Table 3
Results of the sensitivity analysis for the significant main analyses. Percentage difference (95% CI) in relative average telomere length and mitochondrial DNA content for an IQR increment in chronic $PM_{2.5}$ and recent BC and NO_2. Estimates were adjusted for sex, age, BMI, socioeconomic status, passive smoking, season of examination, examination, apparent temperature and the random effect of school and subject.

	n	Telomere length			Mitochondrial DNA content		
		% Difference	95% CI	p-value	% Difference	95% CI	p-value
Main model chronic $PM_{2.5}$		−8.9	−15.4 to −1.9	0.014	−12.7	−21.7 to −2.6	0.016
+ Ethnicity	194	−8.7	−15.3 to −1.5	0.019	−12.6	−21.7 to −2.3	0.018
+ Physical activity	189	−9.2	−15.8 to −2.0	0.013	−13.2	−22.5 to −2.9	0.014
+ Means of transportation to school	195	−7.3	−14.1 to 0.08	0.053	−11.7	−21.2 to −1.1	0.031
+ Neighbourhood income	197	−8.8	−15.4 to −1.7	0.017	−14.1	−23.2 to −4.0	0.0078
Main model recent BC		−6.2	−10.6 to −1.6	0.0091	0.3	−6.4 to 7.4	0.94
+ Ethnicity	194	−5.9	−10.3 to −1.3	0.014	0.8	−5.9 to 8.0	0.81
+ Physical activity	189	−7.1	−11.7 to −2.3	0.0045	−1.4	−8.4 to 6.1	0.70
+ Means of transportation to school	195	−5.9	−10.4 to −1.2	0.015	0.2	−6.5 to 7.5	0.95
+ Neighbourhood income	197	−6.1	−10.5 to −1.5	0.017	−0.1	−6.7 to 7.0	0.99
Main model recent NO_2		−6.4	−11.8 to −0.7	0.029	−6.8	−14.5 to 1.6	0.11
+ Ethnicity	194	−6.1	−11.6 to −0.3	0.039	−5.9	−13.6 to 2.6	0.17
+ Physical activity	189	−8.0	−13.6 to −2.0	0.097	−8.8	−16.8 to 0.1	0.048
+ Means of transportation to school	195	−6.3	−11.8 to −0.6	0.033	−6.8	−14.5 to 1.6	0.11
+ Neighbourhood income	197	−6.3	−11.7 to −0.6	0.031	−6.9	−14.5 to 1.4	0.10

children between 11 and 18 years old living in California. They reported an inverse association between annual polycyclic aromatic hydrocarbons (PAH), as a marker of traffic-related air pollution, and TL from peripheral blood cells (Lee et al. 2017). Clemente et al. (2019) showed that an increase in 1-year childhood NO_2 and $PM_{2.5}$ exposure was inversely associated with leukocyte TL in 1396 European children of 8 years old (Clemente et al. 2019). Thirdly, in 200 preschool children higher annual concentrations of outdoor particulate matter were associated with a lower leukocyte TL (Moslem et al. 2020). In contrast, Walton et al. (2016) showed a longer salivary TL with higher annual NO_x, NO_2, $PM_{2.5}$, and PM_{10} exposures in 333 children (8–9 years old), possibly caused by an inflammatory response in the lungs which might increase the production and circulation of leukocytes (Walton et al. 2016). Oxidative stress due to air pollution could also induce apoptosis of the leukocytes, which are replaced by new cells who have undergone less cell divisions and consequently have longer telomeres (Thiede et al. 2000). However, the other studies follow our hypothesis indicating that ROS-induced damage to telomeres is ineffectively repaired, resulting in telomere shortening (Martens and Nawrot 2016).

Secondly, we found an inverse association between chronic $PM_{2.5}$ exposure and buccal mtDNAc. This association has been previously observed in a sub-set of children of the COGNAC study where it was also found that mtDNAc modified the effect of $PM_{2.5}$ on the heart rate variability (Saenen et al. 2019). Previous studies on newborns and adults showed comparable results. In a study by Janssen et al. (2012), a 10 μg/m^3 increment in PM_{10} exposure during the last month of pregnancy was associated with a 16.1% (95% CI: −25.2, −6.0%, $p = 0.003$) lower placental mtDNAc (Janssen et al. 2012). Similarly, an inverse association was found between annual $PM_{2.5}$ exposure and leukocyte mtDNAc in 166 nonsmoking elderly participants (Pieters et al. 2015). Consistent with the previous studies, in 2758 healthy women lower mtDNAc was observed in relation with annual exposure to $PM_{2.5}$ (Wang et al. 2020). In contrast, recent PM exposure in 63 healthy steel workers was positively associated with leukocyte mtDNAc (Hou et al. 2010). Finally, in a study in Massachusetts, US including 675 older men with a lower average recent exposure of BC (1.0 μg/m^3) compared to our study, both recent and sub-chronic exposure were associated with higher leukocyte mtDNAc (Zhong et al. 2016). These inconsistent results indicate that mtDNAc may react differently under the influence of different air pollutants, concentrations and personal characteristics.

As shown by a systematic review and meta-analysis by Gardner et al. (2014), telomere length is generally longer in women compared with men (Gardner et al. 2014) and this difference is already apparent from birth onwards (Martens et al. 2017). As for TL, also sex-related differences in mtDNAc have been observed (Knez et al. 2016). After stratification for sex, we observed significant associations between TL and air pollution for boys but not for girls whereas the opposite was found for mtDNAc. Similarly, maternal exposure to $PM_{2.5}$, PM_{10}, CO and SO_2 during the third trimester of pregnancy was stronger associated with newborn cord blood TL in boys comparted with girls (Song et al. 2019). However, also stronger effects in girls have been reported (Moslem et al., 2020; Rosa et al., 2019) and also no effect-modifications by sex were found in literature (Clemente et al. 2019). For mtDNAc, research showed that the association with short-term exposure to elemental carbon (EC) and PM_{10} was stronger in men compared to women (Hou et al. 2013) whereas another study found no effect modification by sex in the association between in *utero* exposure and newborn mtDNAc (Hu et al. 2020).

Besides the interaction of sex, we also assessed the association between the biomarkers and chronological age as they are known to be part of the core axis of ageing (Martens and Nawrot, 2016; Sahin and DePinho, 2012). In a recent study in adults where the age ranged between 19 and 79 years, a one-year increment in age was associated with a 0.4% (95% CI: −0.6 to −0.2%, $p = 0.0012$) and a 0.7% (95% CI: −0.9 to −0.4%, $p < 0.0001$) shorter leucocyte TL in women and men respectively (Martens et al. 2018). In our study, we found a negative non-significant trend between age and TL. Due to the small age-range in our study, we might not be able to identify the effect of chronological age. For mtDNAc, we found that and increase in age was associated with a higher mtDNAc. As more recent evidence shows a curvilinear association between mtDNAc and age, indicating that mtDNA content slightly increased from the age of 18 until the fifth decade of life and declined in older subjects, this might explain the positive association (Knez et al. 2016).

We acknowledge some limitations of this study. Firstly, DNA was sampled with buccal swabs because this is a common, non-invasive method compared with drawing blood, especially because children were the subjects. Although there is an absolute difference in TL between different somatic tissues, research showed a correlation between different tissue types (Demanelis et al., 2020; Stout et al., 2017) and a similar rate of age-dependent telomere attrition across different tissues during adulthood (Daniali et al. 2013). Also, for buccal TL, a high intra-individual correlation has been found with blood TL ($r = 0.74$, $p < 0.0001$) (Gadalla et al. 2010). A disadvantage of the use of buccal swabs is the lower quality of the obtained DNA (Hansen et al. 2007). This decreased the efficiency of the analysis and resulted in a loss of data. A second disadvantage is that the oral cell composition might be influenced by poor oral hygiene or infection (Shalev 2012). Nevertheless, buccal cell telomeres are possibly less affected by regulatory factors as is the case for leukocytes because of a more diverse cell population in blood (Shalev 2012). Also, a standardized protocol was used in which the children rinsed their mouth thrice with water and were not allowed to eat or drink 30 min prior to sampling.

Secondly, air pollution measured at the child's residence is based on modelled data. Pollution measured at their residence is only part of their total exposure to air pollution as exposure at school or during transport are not taken into account. However, proxies of exposure, such as modelled residential $PM_{2.5}$ exposure have recently been shown to be associated with internal exposure to nanosized particles, reflecting exposure to black carbon (Saenen et al. 2017). The strengths of the present study are the use of repeated measures and the fact that we were able to estimate recent and chronic air pollution exposure using a high-resolution spatiotemporal model. Finally, our study is among the first to explore the associations of air pollution with TL and mtDNAc in children.

5. Conclusion

In the present study, we found an inverse association between recent and chronic exposure to air pollution and telomere length in the buccal samples of primary schoolchildren, indicating an accelerated biological ageing. TL was associated with chronic exposure to $PM_{2.5}$ in addition to recent exposure to BC and NO_2. Regarding buccal mtDNAc we found a lower copy number in association with chronic residential exposure to $PM_{2.5}$. These data add to the literature on air pollution-induced effects of biological ageing from early life onwards.

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CRediT authorship contribution statement

Pauline Hautekiet: Methodology, Formal analysis, Writing - original draft, Visualization. **Tim S. Nawrot:** Conceptualization,

Methodology, Writing - review & editing, Supervision, Validation, Funding acquisition, Project administration. **Bram G. Janssen:** Methodology, Writing - review & editing. **Dries S. Martens:** Methodology, Writing - review & editing. **Eva M. De Clercq:** Supervision, Writing - review & editing. **Payam Dadvand:** Writing - review & editing. **Michelle Plusquin:** Writing - review & editing. **Esmée M. Bijnens:** Methodology, Writing - review & editing. **Nelly D. Saenen:** Conceptualization, Investigation, Methodology, Writing - review & editing, Data curation, Project administration.

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.2020.106332.

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