Science of the Total Environment 711 (2020) 135028

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Early life tobacco exposure and children's telomere length: The HELIX project

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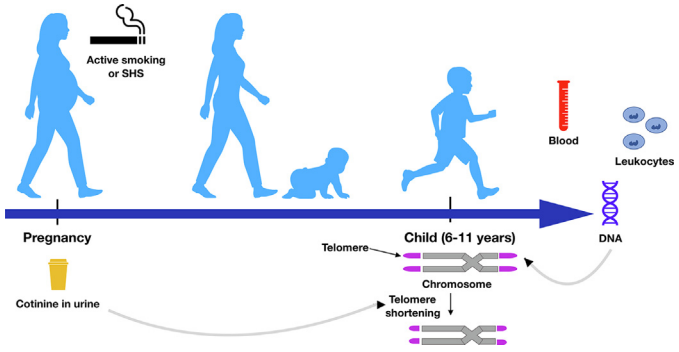
HIGHLIGHTS

- Studies on prenatal exposure to tobacco and children's LTL and mtDNA are limited.
- Maternal cotinine during pregnancy is associated with shorter LTL in children.
- Global tobacco exposure is linked to mitochondrial DNA increase in children.
- Maternal smoking during pregnancy may induce biological aging from an early age.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 January 2019
Received in revised form 14 October 2019
Accepted 16 October 2019
Available online 20 November 2019

ABSTRACT

Telomere length and mitochondrial DNA content are considered biomarkers of cellular aging, oxidative stress, and inflammation, but there is almost no information on their association with tobacco smoke exposure in fetal and early life. The aim of this study was to assess whether prenatal and childhood tobacco exposure were associated with leukocyte telomere length (LTL) and mitochondrial DNA (mtDNA) content in children. As part of a multi-centre European birth cohort study HELIX (Human Early-Life Exposome) (n = 1396) we assessed maternal smoking status during pregnancy through

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https://doi.org/10.1016/j.scitotenv.2019.135028

Editor: Wei Huang

Keywords:
Tobacco exposure
Leukocyte telomere length
Mitochondrial DNA content
Pregnancy
HELIX

questionnaires, and through urinary cotinine levels that were then used to classify women as not exposed to smoking (<10 μg/L), exposed to secondhand smoke (SHS) (10–50 μg/L) and active smokers (>50 μg/L). When the children were around 8 years of age (range: 5.4–12.0 years), childhood SHS tobacco smoke exposure was assessed through an extensive questionnaire and through measurements of urinary cotinine (<3.03 μg/L non-detected, >3.03 μg/L detected). Leukocyte mtDNA content and LTL were measured in the children at 8 years employing real time polymerase chain reaction (qPCR). Effect estimates were calculated using multivariate linear regression models for prenatal and childhood exposures adjusted for potential confounders. Maternal cotinine levels indicative of SHS exposure during pregnancy were associated with a decrease of 3.90% in LTL in children (95% CI: −6.68, −0.91), compared with non-smoking, whereas the association for maternal cotinine levels indicative of active smoking did not reach statistical significance (−3.24%; 95% CI: −6.59, 0.21). Childhood SHS tobacco exposure was not associated with LTL in children. Global SHS exposure during childhood was associated with an increase of 3.51% (95% CI: 0.78, 6.27) in mtDNA content. Our findings suggest that tobacco smoke exposure during pregnancy, even at SHS levels, may accelerate telomere shortening in children and thus induce biological aging from an early age.

1. Introduction

Maternal tobacco smoking during pregnancy has been associated with adverse maternal and fetal outcomes (Gibbs et al., 2016; Windham et al., 2000). The prevalence of tobacco smoking among women is higher in Europe than many regions in the world (e.g., 19% in Europe vs. 2% in Africa) (WHO, 2015). Despite the decrease of maternal active smoking during pregnancy reported for many European countries (Curtin and Matthews, 2016; Reitan and Callinan, 2017), pregnant women are still exposed to secondhand tobacco smoke (SHS) in public places (Fu et al., 2016). According to the World Health Organization (WHO), half of the children in the world are exposed to SHS in public places (WHO, 2009), and many adults are unaware or unconvinced of the adverse effects of SHS exposure (Hall et al., 2014). Cigarette smoke contains over 4,000 different compounds, and many of them are either toxic or carcinogenic (Fowles and Dybing, 2003). Early life exposure to SHS has been associated with adverse cardiovascular risk profile later in life (Dixit et al., 2016), asthma (Butz et al., 2011) and reduced lung function (Henderson et al., 2010).

Telomere length and mitochondrial DNA (mtDNA) content are biomarkers related to cellular aging, oxidative stress and inflammation, and may therefore be of interest to elucidate tobacco smoke mechanisms in the pathway from tobacco smoke exposure to disease or as exposure biomarkers for smoking (Brunst et al., 2015; Pieters et al., 2015). Telomeres consist of non-coding nucleotide sequences (telomeric DNA) forming a nucleoprotein complex at the end of chromosomes that protect against coding DNA erosion, genomic instability and end-to-end fusions. Telomere length shortens with each cell division and it is considered a biomarker of cellular aging and shorter telomeres have been associated with age-related diseases such as cardiovascular disease (Sharifi-Sanjani et al., 2017; Toupance et al., 2017), type 2 diabetes (Sethi et al., 2016) and increased mortality (Batsis et al., 2018). Telomeric DNA are tandem TTAGGG sequences highly sensitive to reactive oxygen damage due to the high guanine content (−GGG) (Houben et al., 2008; Von Zglinicki 2002). Tobacco smoke exposure induces oxidative stress (Cao et al., 2016) and some epidemiologic studies have shown associations between active smoking (either former or current) and shorter telomere length in adults (Astuti, 2017; Wulaningsih, 2016; Zhang et al., 2016). However, there is scarce epidemiologic evidence examining prenatal and childhood exposure to tobacco and telomere length in children. Higher telomere attrition occurs in childhood compared with adulthood (Hjelmborg et al., 2015) and telomere length might be more sensitive to tobacco smoke exposure in early life (Whiteman et al., 2017). To the best of our knowledge, there are only two studies in children focused on prenatal tobacco exposure and telomere length shortening (Ip et al., 2017; Theall et al., 2013); however, these studies were both small (less than 200 subjects).

Mitochondria contain their own genome (MtDNA), a double-stranded circular molecule of 16 569 base pairs, inherited from the maternal line (Taanman 1999). As with telomeres, mitochondrial DNA is susceptible to oxidative damage due to the lack of protective histones and high mutation rate (Passos et al., 2007). Previous studies in adults have shown that tobacco smoke exposure can increase mitochondrial DNA damage and deletions in alveolar macrophages of the lung (Ballinger et al., 1996) and induce mitochondrial changes such as mtDNA content and heteroplasmy in buccal cells (Tan et al., 2008). However, there are few studies relating this to early life-exposure and the results still conflicting; prenatal tobacco exposure has been linked to lower mtDNA content in placental tissue in two small studies (Bouhours-Nouet et al., 2005; Janssen et al., 2017).

Environmental factors might have higher impact on telomere length in early life than in adulthood because telomere length is mainly determined at birth (Bijnens et al., 2017) and high attrition occurs in early childhood (Hjelmborg et al., 2015), whilst telomere length declines at a slower rate through adulthood (Benetos et al., 2013). Similarly, mitochondrial function is essential for developmental process and the mitochondria may be especially vulnerable in early life (Brunst et al., 2015). In order to gain mechanistic insights into the etiology of age-related disease from the earliest life periods, it is thus important to study the link between early life tobacco exposure and telomeres and mtDNA in childhood. The aim of this study was thus to assess prenatal and childhood exposure to tobacco smoke in relation to leukocyte telomere length (LTL) and mtDNA content at age of 8 years in children from a multi-centre birth cohort study in six European countries.

2. Methods

2.1. Subjects

This study used data collected as part of the Human Early-Life Exposome (HELIX) project. The characteristics of the HELIX cohort and inclusion criteria were described previously (Maitre et al., 2018; Vrijheid et al., 2014). HELIX estimated prenatal and childhood exposure to a broad range of chemical and physical exposures in six existing birth cohort studies in Europe: BiB (Born in Bradford; United Kingdom) (Wright et al., 2013), EDEN (Étude des Déterminants pré and postnatals du développement et la santé de l' ENfant, France) (Heude et al., 2016), INMA (INfancia y Medio Ambiente; Spain) (Guxens et al., 2012), KANC (Kaunus Cohort;

Lithuania) (Grazuleviciene et al., 2009), MoBa (Norwegian Mother and Child Cohort Study; Norway) (Magnus et al., 2016), and Rhea (Greece) (Chatzi et al., 2009). The entire six cohorts comprise 32,000 mother–child pairs, and biomarkers were measured in a subcohort of 1301 mother–child pairs. The 1301 mother–child pairs were nested within the entire cohorts by selection of around 200 pairs from each cohort: eligibility criteria were previously described (Maitre et al., 2018). We analyzed data from HELIX sub-cohort with LTL and mtDNA measurements (N = 1190) and we added extra mother–child pairs from the INMA cohort with available information for prenatal and postnatal tobacco exposure (N = 206). The analytical population for this study included a maximum of 1396 mother–child pairs.

2.2. Prenatal tobacco exposure

Each cohort assessed trimester-specific prenatal tobacco smoke exposure through questionnaires with the mother. All the cohorts collected information about active smoking and SHS exposure at first, second and third trimester of pregnancy. We constructed a variable that included both maternal SHS exposure and active smoking exposure as follows: "non-smoker" (non-smoker at the beginning of and during pregnancy with no reported SHS exposure); "SHS" (non-smoker at the beginning of and during pregnancy with reported SHS exposure at home, work, or other regularly places); "active smoker" (smoker at the beginning of and during pregnancy with or without passive smoking exposure) (Robinson et al., 2016). These exposure categories were compared with cotinine measured in urine samples collected at different time points during pregnancy.

As part of the HELIX project cotinine concentrations were measured in maternal urine samples collected during pregnancy in cohorts BiB (third trimester), EDEN and MOBA (second trimester), and RHEA (first trimester) using an automated immunoassay method (IMMULITE 2000 XPi Immunoassay System from Siemens Healthineers) at Norwegian Institute of Public Health (NIPH, Oslo). For INMA, already measured cotinine concentrations were used, urinary cotinine was measured by competitive enzyme immunoassay (EIA) using commercial EIA microplate test kits (OraSure Technologies, Inc, Bio-Rad), as previously described (Aurrekoetxea et al., 2013). In KANC no maternal urine samples were collected during pregnancy so KANC was excluded from maternal pregnancy cotinine analyses.

Based on urinary cotinine, we constructed three categories of maternal smoking status as follows: non-smokers (<10 μg/L), below the limit of quantification (LOQ) for cotinine at Norwegian Institute of Public Health (NIPH) (Haug et al., 2018); SHS exposure (10.0–50 μg/L) and active smokers (>50 μg/L) (Aurrekoetxea et al., 2013).

2.3. Childhood tobacco exposure

Children from the HELIX subcohort were examined in a common follow-up at age 6–11 years across the 6 cohorts (Maitre et al., 2018). Interviews with the mothers during the visit used a computer-aided version of a common standardized questionnaire developed for HELIX. The standardized smoking questionnaire aimed to collect information on maternal smoking status, smoking by parents, smoking by partners, and smoking by other people living with the child. Information on the child's exposure to SHS indoors was also gathered, including other homes, bars/restaurants and other places. Parents reported the child's exposure to SHS indoors as dichotomous (yes, no) and the frequency (times per week) by which the child visited these places. We dichotomized this information into a global SHS exposure variable as "no SHS exposure" (no exposure at home or in other places) and "SHS exposure" (exposure in at least one place). In our analyses we included "parental smoking" (none, one, both parents) and "global SHS exposure".

As a biomarker of SHS exposure we measured cotinine concentrations in urine samples from the children collected at the time of the follow-up visit. HELIX collected two urine samples (one before bedtime and one first morning void) to better capture short-lived biomarkers (Vrijheid et al., 2014). A pool of these two urines was employed to measure cotinine concentrations using an automated immunoassay method (IMMULITE 2000 XPi Immunoassay System from Siemens Healthineers) at Norwegian Institute of Public Health (NIPH, Oslo). We employed in our analyses the cotinine variable as dichotomous: below or above the LOQ (3.03 μg/L). The extra-INMA children (N = 206) did not have information about urinary cotinine levels.

2.4. DNA extraction and measurement of mtDNA content and telomere length in children's blood

2.4.1. Blood collection and DNA extraction

DNA was obtained from buffy coat collected in EDTA tubes in the HELIX subcohort and INMA children at the follow-up visit at age 6–11 years old. Briefly, DNA was extracted using the Chemagen kit (Perkin Elmer) in batches of 12 samples. Samples were extracted by cohort and following their position in the original boxes. DNA concentration was determined in a NanoDrop 1000 UV–Vis Spectrophotometer (ThermoScientific) and with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies). DNA integrity was assessed by agarose-gel electrophoresis.

2.4.2. Average relative mtDNA content measurement

Relative mtDNA content was measured in DNA extracted from buffy coat by determining the ratio of two mitochondrial gene copy numbers (mitochondrial encoded NADH dehydrogenase subunit 1 (MT-ND1) and mitochondrial forward primer for nucleotide 3212 and reverse primer from nucleotide 3319 (MTF3212/R3319)) to one single-copy nuclear control gene (acidic ribosomal phosphoprotein P0 (RPLP0)) using quantitative real-time PCR (qPCR). MtDNA and single copy-gene reaction mixture and PCR cycles used can be found in Janssen et al. (2012). All measurements were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well format. On each run, a 6-point serial dilution of pooled DNA was run to assess PCR efficiency as well as eight inter-run calibrators to account for the inter-run variability. Relative mtDNA content was calculated using qBase software (Biogazelle, Zwijnaarde, Belgium). Coefficient of variation (CV) within triplicates was 0.72% for mitochondrial genes and 0.43% for the single-copy gene.

2.4.3. Average relative telomere length measurement

Average relative telomere length was measured by a modified qPCR protocol as described previously (Cawthon 2009). Telomere and single copy-gene reaction mixture and PCR cycles used can be found in Martens et al. (2016). All measurements were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well format. On each run, a 6-point serial dilution of pooled DNA was run to assess PCR efficiency as well as eight inter-run calibrators to account for the inter-run variability. Relative telomere lengths were calculated using qBase software (Biogazelle, Zwijnaarde, Belgium) and were expressed as the ratio of telomere copy number to single-copy gene number (T/S) relative to the average T/S ratio of the entire sample set. We achieved CV's within triplicates of the telomere runs, single-copy gene runs, and T/S ratios of 0.84%, 0.43%, and 6.4%, respectively.

2.4.4. Blood cell type composition

White blood cell proportions (CD4+ and CD8+ T-cells, natural killer (NK) cells, monocytes, eosinophils, neutrophils, and B-cells) were estimated using Houseman algorithm (Houseman et al., 2012) and the Reinius reference panel (Reinuis et al., 2012) from raw methylation data.

2.4.5. Exposure to particulate matter with aerodynamic diameter ≤2.5 μm ($PM_{2.5}$) during pregnancy

We assessed prenatal $PM_{2.5}$ exposure as previously described (Clemente et al., 2019). Briefly, prenatal $PM_{2.5}$ exposure was estimated using land use regression (LUR) models, temporally adjusted to measurements made in local background monitoring stations and averaged over trimester 1, trimester 2, trimester 3 and whole pregnancy period. For this manuscript, $PM_{2.5}$ averaged over the pregnancy period was used as a potential confounder for the association between smoking exposure during pregnancy and LTL in children.

2.5. Covariate information

As part of their examinations the six cohorts collected detailed questionnaire information on maternal age at birth, maternal education, maternal marital status, maternal ethnicity, mode of delivery, and parity. Maternal pre-pregnancy BMI was calculated from reported pre-pregnancy weight and measured or reported height at the first visit. Measured height was used for BIB and EDEN and self-reported height for the rest of the cohorts. Parents or tutors reported seven possible answers for child ethnicity including White European, White not European, Native American, African, Asian, or a different minority. Child ethnicity was gathered into two categories White European or Other. Child weight and height at age 8 years were measured using a common harmonized protocol across the six cohorts (without shoes and in light clothing). Age- and sex- specific child body mass index (BMI) z-scores were calculated based on the WHO standard reference (de Onis et al., 2007; de Onis et al., 2009).

2.6. Statistical analyses

We tested normality of the variables of interest by computing Shapiro-Wilk and Skewness/Kurtosis test. mtDNA content and telomere length were log 10 transformed to obtain normal distributions. Continuous variables were expressed as median and interquartile range, and categorical variables as frequencies and percentages. ANOVA or *X2* test was used to evaluate differences in sociodemographic characteristics according to smoking status. We calculated Spearman's correlation coefficients to assess the relationship of LTL or mtDNA content with age or BMI. We used simple linear regression models to estimate associations of LTL and mtDNA content with prenatal or postnatal tobacco exposure. We then fitted multiple regression models adjusted for potential confounders, and we reported the percentage of change in the estimates and 95% confidence intervals (CIs).

We imputed exposures and confounders assuming missing at random (MAR) (Royston and White, 2011). Maternal cotinine levels were not imputed for KANC cohort because values were missing for the entire cohort. For HELIX sub-cohort, missing values in the exposure variables ranged from 1.3% (parents smoking) to 16.2% (child cotinine levels) of participants. The missing values for the potential confounders ranged from 0.2% (child's ethnicity) to 41% (breastfeeding). The missing values were imputed using the method of chained equations (White et al. 2011), using the mice package in R (Van Buuren and Groothuis-Oudshoorn, 2011). The following points were taken into account 1) the imputation models included no more than 15–20 variables (Van Buuren and Groothuis-Oudshoorn, 2011) and *quickpred* function was used to reduce the number of predictors; 2) predictive mean matching was used to imputed continuous covariates and logistic or multinomial regression was employed to impute binary or categorical exposures, respectively; 3) Variables that were functions of others (e.g., BMI is a function of weight of weight and height) were not imputed and were calculated after imputation; 4) M = 20 imputed datasets were created for each analyses (White et al., 2011). After imputation, we conducted diagnostics that included comparisons of the imputed and non-missing observations using density plots and stripplots (Van Buuren and Groothuis-Oudshoorn, 2011). If the variables included in the imputation process were not flagged (Stuart et al., 2009) and imputations seemed plausible, we included the predictors in the imputation model.

We selected confounders based on biological plausibility, their influence on model fit (adjusted R-squared) or their effect on the association between tobacco exposure and TL and mtDNA content. All linear regression models adjusted for the following set of confounders: cohort (BiB, EDEN, INMA, KANC, MOBA, and RHEA), child's sex (male, female), child's age (continuous in days), child's ethnicity two categories [White-European, Other], maternal age at birth (continuous in years), maternal pre-pregnancy BMI (continuous, in kg per meter squared), maternal education (low, middle, high), qPCR batch and cell type proportions (% of natural killer cells, B-cell, CD4T, CD8T, monocytes and granulocytes).

We performed the following sensitivity analysis for associations that reached statistical significance: i) excluding one cohort at a time to determine the influence of a particular cohort, ii) further adjustment for parental smoking status at child age (neither, one or two parents), iii) further adjustment for birth weight (g, as continuous variable), iv) further adjustment for $PM_{2.5}$ levels during pregnancy ($\mu g/m^3$, as continuous variable), v) further adjustment for breastfeeding (never/ever), vi) gestational age at delivery (weeks, as continuous variable) and v) forest plot to show cohort by cohort results.

Because the categorization of smoking status according to cotinine levels is not clear cut, we performed additional sensitivity analyses employing cotinine concentrations as continuous variable, two categories of cotinine (< or >= 10.0 μg/L), and tertiles of cotinine levels (<0.1 μg/L; 0.1–17.3 μg/L; >17.3 μg/L) .

All the analyses were performed using RStudio software 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). All reported P-values are two sided and deemed significant at $\alpha = 0.05$.

3. Results

3.1. Characteristics of the study population

The mothers in our cohort had a median age at delivery of 31 years and almost half of the mothers were highly educated (Table 1). On average, 53.9% of the participants were boys and the median age of the children was 8.2 years. The majority of the children were White European (87.4%) and few of them from other origin (12.4%). Women who actively smoked during pregnancy tended to be younger and less educated than women who did not smoke during pregnancy, and they had lower parity (Table 1). Children of actively smoking women had lower birthweight, older age, higher BMI, and were more likely to be of European background.

The median and IQR for telomere length and mtDNA content were 1.0 (0.88–1.13) and 1.0 (0.89–1.21), respectively. Shorter LTL in children was correlated with higher child BMI (r = −0.081; P-Value = 0.003) and we observed shorter LTL in boys in comparison with girls (0.98 vs. 1.02, respectively). MtDNA content levels increased with the age of the children (r = 0.16; P-Value < 0.001)

Table 1
Characteristics of the study population (N = 1396) and stratified by maternal smoking status assessed by questionnaire during pregnancy.

Characteristics	All subjects (N = 1396)	No active smoking during pregnancy (N = 648)	SHS exposure (N = 433)	Active Smoking (N = 220)	P-Value
Maternal					
Maternal age (years)	31.0 (27.0–34.0)	31.0 (28.0–34.0)	30.0 (27.0–33.0)[a]	30.0 (26.5–33.0)[a]	0.001
Missings (N, %)	15 (1.1)	2 (0.3)	0 (0.0)	1 (0.5)	
Maternal education					
Low	219 (15.7)	79 (12.2)*	66 (15.2)	57 (25.9)*	<0.001
Middle	480 (34.4)	166 (25.6)	181 (41.8)	115 (52.3)	
High	643 (46.1)	382 (59.0)*	183 (42.3)	43 (19.5)*	
Missings (N, %)	54 (3.9)	21 (3.2)	3 (0.7)	5 (2.3)	
Pre-pregnancy BMI (kg/m^2)	23.8 (21.3–27.2)	23.8 (21.3–26.8)	23.9 (21.6–26.9)	23.5 (21.2–27.8)	0.686
Missings (N, %)	30 (2.2)	7 (1.1)	2 (0.5)	1 (0.5)	
Mode of delivery					
Vaginal	928 (66.5)	469 (72.4)*	238 (55.0)*	153 (69.5)	<0.001
C-section	250 (17.9)	78 (12.0)*	110 (25.4)*	48 (21.8)	
Missings (N, %)	218 (15.6)	101 (15.6)	85 (19.9)	19 (8.6)	
Parity					
1	635 (45.5)	280 (43.2)*	193 (44.6)	126 (57.3)*	0.002
2	498 (35.7)	238 (36.7)	161 (37.2)	71 (32.3)	
≥3	228 (16.3)	121 (18.7)*	72 (16.6)	21 (9.5)*	
Missings (N, %)	35 (2.5)	9 (1.4)	7 (1.6)	2 (0.90)	
Prenatal PM2.5 (μg/m^3)	15.0 (13.5, 16.9)	14.7 (13.0, 17.2)	15.1 (14.0, 16.6)[a]	15.1 (13.9, 16.3)	0.023
Missings (N, %)	89 (6.4)	16 (2.5)	5 (1.2)	7 (3.2)	
Child					
Birth weight (g)	3360 (3040–3670)	3410 (3100, 3720)	3340 (3040, 3690)	3220 (2900, 3500)[a,b]	<0.001
Missings (N, %)	12 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	
Breastfeeding					
Never	105 (7.5)	41 (6.3)	34 (7.9)	30 (13.6)	0.067
Ever	719 (51.5)	258 (39.8)	304 (70.2)	145 (65.9)	
Missings (N, %)	572 (41.0)	349 (53.9)	95 (21.9)	45 (20.5)	
Sex					
Male	753 (53.9)	347 (53.5)	237 (54.7)	120 (54.5)	0.726
Female	643 (46.1)	301 (46.5)	196 (45.3)	100 (45.5)	
Missings (N, %)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Age (years)†	8.2 (6.5–9.1)	8.3 (6.7–9.2)	6.9 (6.4–8.9)[a]	8.8 (6.7–9.6)[b]	<0.001
z-BMI	0.4 (−0.4, 1.2)	0.2 (−0.5, 1.0)	0.5 (−0.3, 1.4) [a]	0.8 (−0.1, 1.6)[a]	<0.001
Missings (N, %)	14 (1.0)	5 (0.8)	5 (1.2)	1 (0.5)	
BMI categories					
Thin (Grades 1–3)	103 (7.4)	56 (8.6)	32 (7.4)	12 (5.5)	<0.001
Normal weight	980 (70.2)	483 (74.5)*	284 (65.6)*	139 (63.2)*	
Overweight	215 (15.4)	84 (13.0)*	70 (16.2)	49 (22.3)*	
Obese	84 (6.0)	20 (3.1)*	42 (9.7)*	19 (8.6)	
Missings (N, %)	14 (1.0)	5 (0.8)	5 (1.2)	1 (0.50)	
Ethnicity					
White European	1220 (87.4)	549 (84.7)*	390 (90.1)*	203 (92.3)*	0.001
Other	173 (12.4)	99 (15.3)*	42 (9.7)*	16 (7.3)*	
Missings (N, %)	3 (0.2)	0 (0.0)	1 (0.2)	1 (0.45)	

Continuous variables expressed as median and 25-75th percentile; categorical variables were described as N(%).
ANOVA with Tukey multiple pairwise-comparisons test for continuous variables.
X^2 test with Pearson standardized residuals for categorical variables.
*P-Value <0.05.
[a]P-Value < 0.05 compared to no active smoking during pregnancy.
†No missing values.
$PM_{2.5}$. Particulate matter with aerodynamic diameter ≤2.5 μm.
[b] P-Value <0.05 compared to SHS exposure during pregnancy.

and mothers with higher education had higher mtDNA content. Mitochondrial DNA content decreased with the increasing maternal BMI (r = −0.13; P-value < 0.001). Children who were born by C-section had lower mtDNA content (0.97 vs. 1.02 for vaginal delivery; P-Value < 0.001) and we observed higher mtDNA content in White European compared with other countries of origin (1.05 vs. 0.88, respectively) (P-Value < 0.0001).

Overall, 46.4% of the mothers reported that they did not smoke actively and were not exposed to SHS exposure during the pregnancy, whereas 31.0% reported SHS exposure and 15.8% reported active smoking during pregnancy (Table 2). The smoking status categories from questionnaire data were significantly related to respective urinary cotinine levels in the mothers. Median cotinine concentrations for non-smokers, SHS exposure and active smokers were 21.6, 113.4 and 1747.4 μg/L, respectively (P < 0.001) (Fig. 1). According to maternal cotinine concentrations in urine, 23.0% and 16.8% were classified as exposed to SHS and active smokers, respectively. Smoking status varied substantially between cohorts, with the percentage active smokers ranging from 23.4% in INMA to 4.2% in MOBA. Mothers from RHEA were highly exposed to SHS (69.3%) and only 5.6% of mothers from MOBA reported SHS exposure (not shown). Similarly, children from RHEA and MOBA had the highest and lowest percentages of detectable cotinine levels (37.7 and 0.5%, respectively). Mothers of children with detectable cotinine levels had higher urinary cotinine during pregnancy [(median and IQR): (43.0; 13.4–1402) μg/L] compared to those mothers of children with no-detectable cotinine levels [median and IQR: (0.124; 0.03–15.2) μg/L] (P-Value <0.001).

Table 2
Descriptive statistics of prenatal and postnatal tobacco exposure in HELIX (N = 1396).

Exposure Variables	N (%)	Maternal cotinine concentration (μg/L) or % of detected cotinine levels in children
Prenatal		
Maternal Smoking Status		
Non-Smokers	648 (46.4)	0.08 (0.03, 0.39)
SHS exposure	433 (31.0)	15.3 (0.35, 32.0)
Active Smokers	220 (15.8)	1019 (17.46, 2818)
Missings	95 (6.8)	
Urinary Maternal Cotinine		
Non-Smokers (<10.0 μg/L)	593 (49.7)	0.06 (0.02, 0.14)
SHS Exposure (10.0–50 μg/L)	275 (23.0)	18.9 (13.7, 28.4)
Active Smokers (≥50 μg/L)	201 (16.8)	1360 (154, 2956)
Missings	125 (10.5)	
Postnatal		
Parental smoking		
None	828 (59.3)	27 (12.5)
One	394 (28.2)	106 (49.1)
Both	156 (11.1)	82 (38.0)
Missings	18 (1.3)	1 (0.5)
Global exposure		
No	862 (61.7)	40 (18.5)
Yes	497 (35.6)	170 (78.7)
Missings	37 (2.7)	6 (2.8)
Child Cotinine Levels[b]		
Undetected	954 (68.3)	–
Detected	216 (15.5)	–
Missings	226 (16.2)	

Maternal cotinine levels expressed as median and 25-75th percentile.
[b] Child cotinine levels above or below the detection limit of 3.03 μg/L.

3.2. Prenatal tobacco smoke exposure and telomere length and mitochondrial DNA

Maternal SHS exposure and active smoking reported by questionnaire during pregnancy were associated with a decrease in child LTL (−1.45% [95% CI: −4.15, 1.32] and −1.40% [95% CI: −4.70, 2.01] for SHS exposure and active respectively, compared with non-smokers), but these associations did not reach statistical significance (Table 3). The maternal cotinine category indicative of SHS exposure, as compared with non-smoking, was statistically significantly associated with a shorting in child LTL [−3.86%; (95% CI: −6.68, −0.91); P-value = 0.01] (Table 3). Similarly, we observed that the cotinine category indicating active maternal smoking during pregnancy showed a borderline association with child LTL (−3.24%; (95% CI: −6.59, 0.21); P-value = 0.07) (Table 3). There was no evidence for heterogeneity between cohorts in the analyses of prenatal tobacco exposure (P-Value >0.05). Almost all the sensitivity analyses excluding one cohort at a time (with the exception of RHEA cohort exclusion) revealed significant associations between child LTL and maternal urinary categories indicative of SHS exposure (Table 4). Supplemental Fig. 1 shows the association between SHS exposure and child LTL in analyses stratified by

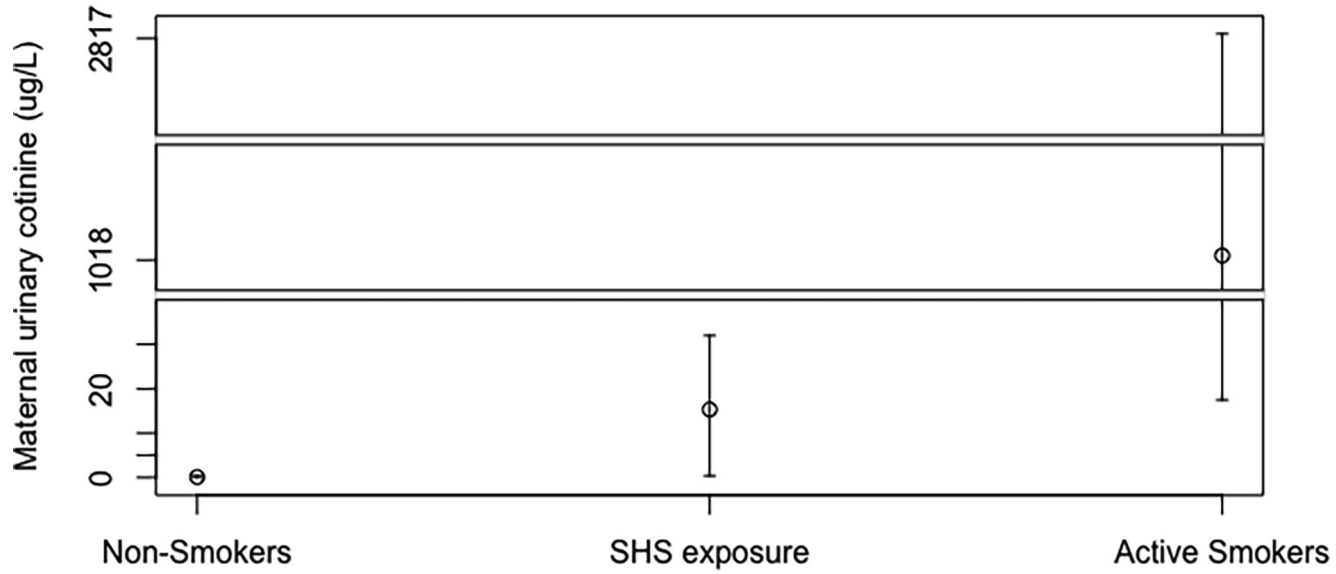

Fig. 1. Maternal cotinine concentrations according to smoking status assessed by questionnaire. Maternal urinary cotinine (μg/L) according to smoking categories reported by questionnaire in pregnant women from the Human Early Life Exposome (HELIX) cohort. SHS, second hand smoke exposure to tobacco. Lines represent median and 95% confidence intervals. ANOVA with Tukey multiple pairwise comparisons to assess cotinine differences according to maternal smoking status: Active Smokers vs. Non-Smokers (P-Value < 0.001) and Active Smokers vs. SHS exposure (P-Value < 0.0001).

Table 3
Associations between prenatal and postnatal tobacco exposure, child's telomere length and mtDNA content in HELIX.

Exposure Variables	Frequency (N)	% change in LTL (95% CI)	P-value	% change in mtDNA content (95% CI)	P-value
Prenatal					
Maternal smoking status					
Non-smokers	648	Reference		Reference	
SHS exposure	433	−1.45 (−4.15, 1.32)	0.30	−1.52 (−4.50, 1.56)	0.33
Active smokers	220	−1.40 (−4.70, 2.01)	0.42	−0.97 (−4.63, 2.83)	0.61
Urinary maternal cotinine[a]					
Non-smokers (<10 μg/L)	593	Reference		Reference	
SHS exposure(10–50 μg/L)	275	**−3.86 (−6.68, −0.91)**	**0.01**	0.15 (−3.04, 3.44)	0.93
Active smokers (≥50 μg/L)	201	*−3.24 (−6.59, 0.21)*	*0.07*	1.60 (−2.16, 5.51)	0.41
Postnatal					
Parental smoking					
None	828	Reference		Reference	
One or both	550	−1.88 (−4.10, 0.41)	0.11	−0.74 (−3.22, 1.81)	0.57
Global exposure					
No	862	Reference		Reference	
Yes	497	−0.56 (−2.93, 1.85)	0.64	**3.51 (0.78, 6.27)**	**0.011**
Child Cotinine Levels					
Undetected (<3.03 μg/L)	954	Reference		Reference	
Detected (>3.03 μg/L)	216	−1.10 (−3.97, 1.87)	0.46	−0.76 (−3.93, 2.50)	0.64

All models adjusted for cohort (BiB, EDEN, INMA, KANC, MOBA, and RHEA), child's sex (male, female), child's age (continuous in days), child's ethnicity (White European, Other), maternal age at birth (continuous in years), maternal pre-pregnancy BMI (continuous, in kg per meter squared), maternal education (low, middle, high), qPCR batch and cell composition (% of natural killer cells, B-cell, CD4T, CD8T, monocytes and granulocytes).
[a]KANC was excluded from this analysis due to the lack of maternal urinary samples. Significant results (P-Value < 0.05) are highlighted in bold and marginally significant results (P-Value < 0.10) are given in italics.

Table 4
Summary of sensitivity analyses of the associations between children telomere length shortening and maternal cotinine concentrations.

	Non-smokers	SHS exposure		Active smokers	
	N	N	% of change in LTL (95% CI)	N	% of change in LTL (95% CI)
5 cohorts-Main Analyses	593	275	**−3.86 (−6.68, −0.91)**	201	*−3.24 (−6.59, 0.21)*
Excluding one cohort at a time					
Excluding BIB (United Kingdom)	479	226	**−4.94 (−8.17, −1.65)**	160	*−3.35 (−7.10, 0.59)*
Excluding EDEN (France)	504	243	**−3.33 (−6.46, −0.064)**	173	−2.88 (−6.61, 0.99)
Excluding INMA (Spain)	435	189	**−4.50 (−7.81, −1.12)**	137	−2.95 (−6.87, 1.03)
Excluding MOBA (Norway)	410	252	**−3.53 (−6.57, −0.38)**	196	*−3.11 (−6.50, 0.43)*
Excluding RHEA (Greece)	544	190	*−3.06 (−6.42, 0.41)*	138	**−4.28 (−8.23, 0.18)**
5 cohorts-further adjustment for parental smoking status	593	275	**−3.66 (−6.59, −0.62)**	201	−2.95 (−6.65, 0.96)
5-cohorts-further adjustment for birth weight	593	275	**−3.86 (−6.72, −0.93)**	201	*−3.11 (−6.44, 0.36)*
5-cohorts-further adjustment for $PM_{2.5}$ levels during pregnancy	593	275	**−3.82 (−6.70, −0.84)**	201	*−3.22 (−6.57, 0.242)*
5-cohorts-further adjustment for breastfeeding	593	275	**−4.39 (−7.96, −0.77)**	201	−3.33 (−7.32, 0.75)
5-cohorts-further adjustment for gestational age at delivery	593	275	**−3.82 (−6.68, −0.87)**	201	*−3.33 (−6.68, 0.136)*

All models adjusted for cohort (BiB, EDEN, INMA, KANC, MOBA, and RHEA), child's sex (male, female), child's age (continuous in days), child's ethnicity (White European, Other), maternal age at birth (continuous in years), maternal pre-pregnancy BMI (continuous, in kg per meter squared), maternal education (low, middle, high), qPCR batch and cell type proportions (% of natural killer cells, B-cell, CD4T, CD8T, monocytes and granulocytes). Parental smoking status at child age (neither, one or both); Birth weight (g, as continuous); $PM_{2.5}$ levels ($\mu g/m^3$, as continuous), breastfeeding (never or ever), gestational age at delivery (weeks, as continuous). Significant results (P-Value < 0.05) are highlighted in bold and marginally significant results (P-Value < 0.10) are given in italics.

cohort. Cohort-specific LTL ranged from 0.20 (95% CI: −6.24, 7.15) in BIB to −6.20 (95% CI: −13.5, 1.79) in EDEN, and from −1.76 (95% CI: −7.90, 4.71) to −7.6 (95% CI: −15.1, 0.64) for the INMA and MOBA cohorts, respectively. No observed heterogeneity was found for LTL shortening, as shown by the Q test of 8.21 (df = 4, p = 0.08). The association between maternal cotinine levels indicative of SHS and shorter LTL in children remained statistically significant after further adjustment for current parental smoking status or birth weight or breastfeeding (Table 4).

Further sensitivity analyses showed that maternal cotinine levels as continuous variable was not associated with child LTL. When we combined the two highest levels of maternal cotinine during pregnancy (SHS [10.0–50 μg/L] and active smoking [>50 μg/L]), cotinine levels (≥10.0 μg/L) were associated with a 3.62% (95% CI: −6.16, −1.02) decrease in LTL compared to low maternal cotinine levels (<10.0 μg/L). Similarly, the highest tertile of maternal cotinine levels during pregnancy (>17.3 vs < 0.1 μg/L) was associated with 3.6% (95% CI: −6.68, −0.40) LTL shortening in children (Table 5). Finally, we did not observe any associations between prenatal smoking exposure and mtDNA content (Table 3).

3.3. Postnatal tobacco smoke exposure and telomere length and mitochondrial DNA.

For postnatal SHS exposure, at child age 6–11 years, we observed a weakly negative association between parental smoking (one or both) and LTL (−1.88%; 95% CI: −4.10, 0.41) but this was not statistically significant (Table 3). Finally, we observed significant associations between global exposure to tobacco during childhood and mtDNA content (vs. no global exposure; 3.51% increase in mtDNA content; 95%CI: 0.78–6.27) (Table 3). Similar results were observed when we conducted sensitivity analyses excluding one cohort at a time (data not shown).

In a model that included both the prenatal cotinine variable and the postnatal parental smoking variable, the association between maternal cotinine levels indicative of SHS and shorter LTL in chil-

Table 5
Summary of sensitivity analyses of the associations between children telomere length shortening and maternal cotinine concentrations.

	N	% of change in LTL (95% CI)	P-Value
Cotinine as continuous (μg/L)	1069	−4.4 e^{-4} (−1.4 e−3, 6.0 e^{-4})	0.387
Cotinine two categories			
Non-Smokers (<10.0 μg/L)	726	Reference	
SHS exposure and Active Smokers (≥10.0 μg/L)	343	**−3.62 (−6.16, −1.02)**	**0.007**
Cotinine as tertiles			
First tertile (<0.1 μg/L)	357	Reference	
Second tertile (0.1–17.3 μg/L)	356	−0.62 (−3.40, 2.31)	0.68
Third tertile (>17.3 μg/L)	356	**−3.55 (−6.68, −0.40)**	**0.03**

All models adjusted for cohort (BiB, EDEN, INMA, KANC, MOBA, and RHEA), child's sex (male, female), child's age (continuous in days), child's ethnicity (White European, Other), maternal age at birth (continuous in years), maternal pre-pregnancy BMI (continuous, in kg per meter squared), maternal education (low, middle, high), qPCR batch and % of natural killer cells, B-cell, CD4T, CD8T, monocytes and granulocytes. Missing covariates and exposures were imputed with the exception of maternal cotinine levels for KANC. Significant results (P-Value < 0.05) are highlighted in bold.

dren remained statistically significant (Table 4), whilst parental smoking was not significant.

4. Discussion

In this large multi-country study, we showed that prenatal exposure to tobacco smoke was associated with a shortening in leukocyte telomere length, a marker of biological aging, in children. This association was most prominent amongst children of mothers with medium cotinine levels indicative of passive smoke exposure during pregnancy. Of note, passive tobacco smoking exposure of the children was only weakly and not significantly associated with LTL shortening. Finally, global exposure to tobacco was associated with an increase in mtDNA content in children.

We observed associations between maternal passive and active smoking during pregnancy and LTL in children. Only passive smoking reached statistically significance, but the results were consistent between children from different cohorts included in our study. Our findings are in line with previous small studies reporting telomere shortening in cord blood and developing lung in fetuses and leukocytes in children associated with tobacco exposure during pregnancy (Ip et al., 2017; Mirzakhani et al., 2017; Salihu et al., 2015; Theall et al., 2013).

Of note, previous studies have reported a linear exposure–response relationship between tobacco exposure and LTL (Ip et al., 2017; Salihu et al., 2015). Our results show a somewhat stronger association for cotinine levels indicative of SHS, than for cotinine levels indicative of active smoking or active smoking reported by questionnaire. One possible explanation of this nonlinearity might be due to saturation phenomenon, whereby relative low levels of exposure are capable of activating relevant biological pathways related to oxidative stress and inflammation (Pope et al. 2011). Almost half of the pregnant women in HELIX reported to be exposed to SHS or being active smokers, which could make this saturation phenomenon a possible explanation for our findings.

Another possible explanation might be some degree of exposure misclassification. Here, we should note that we employed cotinine cutoffs based on LOQ for urinary cotinine (Haug et al., 2018) and a previous study (Aurrekoetxea et al., 2013) and that we found moderate agreement between cotinine measurements and smoking questionnaire answers (kappa factor 0.43). However, we cannot discard some degree of exposure misclassification and it may well be possible that active smokers are included in the SHS cotinine category. There is no current consensus regarding the cut-off point for urinary cotinine levels in pregnant women (Aurrekoetxea et al., 2013). A previous study among pregnant women proposed a cotinine cutoff of 42.3 ng/mL to differentiate mothers who actively smoke during pregnancy compared to non-smokers (Stragierowicz et al., 2013). Therefore, some active smokers might have been classified as SHS exposure in our study.

Our results highlight the long-term effects of tobacco exposure on LTL, in particular they highlight that the biological aging process triggered by tobacco smoke may already start in utero. Nicotine, carbon monoxide and several other substances contained in tobacco smoke are able to cross the placenta and affect fetal development (Banderali et al., 2015, Lisboa et al., 2012); we now also show that they may also predispose children to early aging later in life. Further studies should investigate whether the LTL shortening we observe in children may play a role in later adverse health effects, such as reduced lung function (Gibbs et al., 2016).

We estimate telomere loss based on relative values, since we used real-time PCR method that cannot provide absolute values. Nevertheless, we can estimate the years of life lost in relation to LTL shortening based on the literature. Previous studies have shown that telomere length is on average 8 kb in young adulthood and the annual telomere loss in adult leukocytes is between 32.2 and 45.5 bp (Müezzinler et al., 2013; Nawrot et al., 2004). In our study, we found a reduction in 4% in telomere length associated with prenatal SHS exposure, which would be equivalent to a loss of 320 bp of telomeric DNA indicating 7–9 years of life less in children exposed to tobacco during in-utero life compared to non-exposed children.

The mechanisms that have been proposed to link tobacco exposure and telomere length shortening include oxidative stress and inflammation (Babizhayev and Yegorov, 2011; Huang et al., 2009). Studies conducted in pregnant women showed that tobacco exposure increase ROS production (H_2O_2 and O_2^-) in mononuclear blood cells and advanced oxidative protein products (AOPP) in umbilical cord (Rua Ede et al., 2014). Telomeric DNA are tandem sequences (5′-TTAGGG-3′) highly sensitive to ROS induced damage due to the high guanine content (−GGG). ROS may also induce DNA breakage and telomeric DNA is ineffective in repairing single strand breaks leading to accelerated telomere shortening (Houben et al., 2008).

Natural telomere shortening occurs during each cell division cycle until telomere reaches a critical length and eventually the cell undergoes apoptosis (Babizhayev et al., 2011). Therefore, telomere length shortening is also considered a biomarker of cellular aging (Bekaert et al., 2005). LTL shortening is associated with age-related diseases such as hypertension, cardiovascular events, diabetes, and dementia among others (reviewed in Bojesen, 2013). LTL shortening in children associated with prenatal tobacco smoke exposure might be indicative of early aging or higher risk of age-related diseases.

In addition to telomere length, mitochondria might be a molecular target of tobacco smoke exposure (Ballinger et al., 1996; Janssen et al., 2017; Pirini et al., 2017; Tan et al., 2008).

Mitochondria are both sources of reactive oxygen species (ROS) and also the primary target of ROS (Starkov, 2008). The mitochondrial genome is more susceptible to ROS induced DNA damage than genomic DNA due to the lack of histone protection and the inefficient DNA repair capacity (Passos et al., 2007). Our results showed an increase in leukocyte mtDNA content in children globally exposed to tobacco compared to non-exposed. To the best of our knowledge, no previous epidemiologic study has examined prenatal and postnatal tobacco exposure in relation to leukocytes' mtDNA content in children. Previous epidemiologic studies have been primarily focused on the effects of tobacco exposure during pregnancy on placental mtDNA content. For instance, Bouhours-

Nouet et al. (2005) and Janssen et al. (2017) reported lower mtDNA content in placental tissue from smokers compared to non-smokers [37% and 21.6% decrease, respectively]. On the contrary, Garrabou et al. (2016) found an increase in mtDNA content accompanied with oxidative stress and apopotosis in placenta from smokers (vs. non-smokers). Studies with experimental animals have also showed inconsistent results depending on the specie and organ studied. Tobacco exposure increases mtDNA content in kidney in Balb-c mice (Stangenberg et al., 2015), and decreases mtDNA content in vascular tissue in non-human primate *Macaca mulatta* (Westbrook et al., 2010). The association between global exposure to tobacco and leukocytes' mtDNA content in children must be confirmed in other cohorts. Additionally, we cannot discard the possibility that other mitochondrial DNA markers such as heteroplasmy or epigenetics (Brunst et al., 2015) might be related to tobacco smoke exposure during early life.

The results of our study should be interpreted in light of its strengths and limitations. The strengths of our study include its prospective design, large size, rich covariate data, detailed smoking questionnaire during prenatal and postnatal life combined with urinary cotinine concentrations for both mothers and children. Our study also has some limitations including 1) the short biological half-life of urinary cotinine that might not well reflect cumulative tobacco exposure during pregnancy (Aurrekoetxea et al., 2013); 2) telomere length and mtDNA content were measured only at one time point during childhood; 3) previous studies have shown that paternal age is associated with offspring leukocyte telomere length (Prescott et al., 2012); however, we do not have this information in our cohorts; 4) we measured both biomarkers in leukocytes, a mixture of many cell types, and variations in TL and mtDNA content might be also related to cell composition (Lin et al., 2010); however, we adjusted our analyses for the proportion of natural killer cells, B-cell, CD4T, CD8T, eosinophils, mononuclear cells and neutrophils. 5) Telomere restriction fragment (TRF) analysis is the traditional method to determine telomere length. We used a qPCR protocol that has a higher assay variability compared to the golden standard TRF method (Eastwood et al., 2018). However, we did an inter-laboratory comparison of our relative measure and showed that CV was less than 7%; 6) Due to the high correlation between prenatal and postnatal tobacco exposure it was difficult to disentangle the effects of early life tobacco exposure on TL and mtDNA content.

5. Conclusions and remarks

Our findings suggest that prenatal tobacco smoke exposure during pregnancy, even at SHS levels, may accelerate telomere shortening in children and thus induce biological aging from an early age.

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.

Acknowledgments

We are grateful to all the participating children, parents, practitioners and researchers in the six countries who took part in this study.

Funding

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 308333 – the HELIX project. The Norwegian Mother and Child Cohort Study is supported by the Norwegian Ministry of Health and Care Services and the Ministry of Education and Research, NIH/NIEHS (contract no N01-ES-75558), NIH/NINDS (grant no. 1 UO1 NS 047537-01 and grant no. 2 UO1 NS 047537-06A1). COY was financially supported by Secretary of Science, Technology and Innovation (SECITI-DF; Mexico; Grant No BP15-001). The telomere and mtDNA protocol were developed with grants from FWO (FWO G073315N) and European research council (ERC-2012-StG 310898). Telomere measurements in the INMA cohort were partly funded by a grant grants from Instituto de Salud Carlos III (FIS-PI15/00118).

ISGlobal is a member of the Agency for the Research Centres of Catalonia (CERCA) Programme, Generalitat de Catalunya.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135028.

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