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European-wide studies on pro-inflammatory risk factors in early life and molecular markers of aging

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Abstract

Although universal and unavoidable, aging does not occur in a uniform way. In this dissertation, we assessed the effects of early life exposure to pro-inflammatory risk factors (air pollution and obesity) on mitochondrial DNA (mtDNA) content and telomere length, considered as markers of biological aging, at birth and during childhood. First we observed that an increment in nitrogen dioxide (NO_2) exposure during pregnancy was associated with a decrease in both placental mtDNA content and birth weight and length (**chapter 2 and 3**). Secondly, we showed that the association between prenatal NO_2 exposure and infant growth could be mediated by placental mtDNA content (**chapter 2 and 3**). Thirdly, our study found that increased pre- and postnatal exposure to air pollutants lead to shorter leukocyte telomere length in 8 year old children (**chapter 4**). Finally, we showed that increased obesity indicators were associated with significant shorter telomeres in 8 year old children (**chapter 5**).

Samenvatting

Hoewel veroudering een algemeen en onvermijdelijk proces is, gebeurt het niet via een vast patroon. In dit proefschrift onderzochten we het effect van milieu blootstelling tijdens het vroege leven op het mitochondriaal DNA (mtDNA) inhoud en telomeerlengte, beide beschouwd als biologische merker van veroudering, bij de geboorte en tijdens het vroege leven. Eerst hebben we aangetoond dat een toename in stikstofdioxide (NO2) blootstelling verbonden is met een daling in placentaal mtDNA inhoud en een daling in geboortegewicht- en lengte (**hoofdstuk 2 en 3**). Ten tweede bleek het verband tussen blootstelling aan NO_2 tijdens de zwangerschap en de groei van het kind geïnterfereerd te zijn door placentaal mtDNA inhoud (**hoofdstuk 2 en 3**). Ten derde vonden we dat een verhoogde pre- en postnatale blootstelling aan luchtverontreiniging leidt tot kortere telomeren in 8 jaar oude kinderen (**hoofdstuk 4**). Tenslotte toonden we aan dat een toename in obesitas merkers zorgt voor kortere telomeren in 8 jaar oude kinderen (**hoofdstuk 5**).

Resum

Tot i que és universal i inevitable, l'envelliment no es produeix de manera uniforme. En aquesta tesi, es van avaluar els efectes de l'exposició primerenca a factors de risc proinflamatoris (contaminació de l'aire i obesitat) sobre el contingut d'ADN mitocondrial (mtDNA) i la longitud del telómero, considerats com a marcadors de l'envelliment biològic, en néixer i durant la infància. En primer lloc, vam observar que un increment de l'exposició al diòxid de nitrogen (NO_2) durant l'embaràs es va associar amb una disminució tant del contingut de ADN de la placenta plasmàtica com del pes i la durada del part (**capítols 2 i 3**). En segon lloc, vam mostrar que l'associació entre l'exposició NO_2 prenatal i el creixement infantil podria estar mediada per contingut de ADN de placenta (**capítols 2 i 3**). En tercer lloc, el nostre estudi va descobrir que l'augment de l'exposició pre i postnatal als contaminants atmosfèrics conduir a una menor longevitat de leucòcits en nens de 8 anys (**capítol 4**). Finalment, vam mostrar que un augment dels indicadors d'obesitat es van associar amb telòmers més curts significatius en nens de 8 anys (**capítol 5**).

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List of abbreviations

DOHaD	Developmental Origins of Health and Disease
PM	Particulate matter
So_x	Sulfur oxides
No_x	Nitrogen oxides
O_3	Ozone
NO_2	Nitrogen dioxide
NO	Nitrogen oxide
$PM_{2.5}$	PM2.5 particles with an aerodynamic diameter < 2.5 µm
PM_{10}	PM10 particles with an aerodynamic diameter < 10 µm
UFPs	Ultrafine particles (particles with an aerodynamic diameter < 0.1 µm)
WHO	World Health Organization
BMI	Body mass index
ATP	adenosine-5'-triphosphate
ROS	Reactive oxigen species
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
INMA	INfancia y Medio Ambiente; Environment and Childhood
ENVIR*ON*AGE	ENVIRonmental influence ON early AGEing
CI	Confidence interval
OXPHOS	oxidative phosphorylation
HUSC	Hospital Universitario San Cecilio
MT-ND1	Mitochondrial encoded NADH dehydrogenase subunit 1
MTF3212/R3319	Mitochondrial forward primer for nucleotide 3212 and reverse primer from nucleotide 3319
RPLP0	Acidic ribosomal phosphoprotein P0
ACTB	Beta-actin
qPCR	Quantitative real-time polymerase chain reaction

LUR	Land use regression
GAM	Generalized additive model
DE	Direct effect
IE	Indirect effect
TE	Total effect
IQR	Interquartile range
TRP	Transient receptor potential
RNS	reactive nitrogen species
VIF	Variance inflation factors
LTL	Leukocyte telomere length
HELIX	Human Early Life Exposome
BIB	Born in Bradford
EDEN	Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant
KANC	Kaunus cohort
MoBa	Norwegian Mother and Child Cohort Study
RHEA	Mother Child Cohort study
ISCED	International Standard Classification of Education
GIS	Geographical information system
SD	Standard deviation
TRF	Telomere restriction fragment
OECD	Organization of Economic Cooperation and Development
EU	European Union

Prologue

Although universal and unavoidable, aging does not occur in a uniform way. Aging is a complex physiological phenomenon responsive to both environmental and genetic factors and includes both chronic and acute processes.[1, 2] As our knowledge of developmental biology expands, there is great awareness that health complications in adult life can have their roots in risk factors operative in early life. Aging begins at the very beginning of life, to accelerate middle-age. It is believed that the biological underpinnings of aging may begin before birth.[1] The Barker hypothesis or 'Developmental Origins of Health and Disease' (DOHaD) suggests that small changes in early life environment shape the future probability of the development of age-related diseases.[3] Pro-inflammatory risk factors might influence the aging phenotype through their actions on the primary hallmarks of aging.

Chapter 1.

General introduction

1. Air pollution

Air pollution consists of both gaseous and particulate matter (PM) pollutants, originating from natural (geological dust, forest fires, volcanoes, methane) or anthropogenic sources (fossil fuel burning, refineries/power plants, agriculture, industry, transport). Subdivision can be made in the production processes of these pollutants. Primary pollutants are emitted directly in the air e.g., ash from volcano eruptions, sulfur oxides (SO_x) from industrial processes, nitrogen oxides (NO_x) from vehicle exhaust and toxic metals from metal producing and using factories. After emission into the atmosphere, chemical reactions involving UV-light, ozone (O_3), gaseous pollutants (e.g. SO_x, NO_x) can transform these primary pollutants into secondary pollutants.[4] One example of these secondary pollutants is nitrogen dioxide (NO_2). It's a reactive gas that is mainly formed by oxidation of nitrogen oxide (NO), which is produced by the combustion of fuel oil at high temperatures as occurs in cars, home heating sources and cooking appliances.[5] Once released into the air, NO combines with ozone (O_3) to form NO_2. The latter is not highly soluble and most inhaled NO_2 is retained in the small airways.[6]

Besides this complex mixture of gaseous substances, the atmosphere also contains PM. PM is an airborn mixture of solid and liquid droplets vary in number, size, shape, surface area, chemical composition, solubility, and origin.[7] PM is generally categorized according to its aerodynamic diameter. The majority of PM in ambient air are respirable particles with a diameter lower than 10 µm. The following PM fractions are commonly recognized, based on aerodynamic diameter: 'respirable particles' or the particle fraction between 2.5 and 10 µm, 'fine particles' or the particle fraction less than 2.5 µm, and 'ultrafine particles' (UPFs) or the particle fraction less than 0.1 µm.[8] PM can be produced by anthropogenic sources, predominantly by road traffic including abrasion of brakes and tires. Non-exhaust emission contribute mainly to PM_{10}, while exhaust emissions contribute predominantly to $PM_{2.5}$.[9, 10] Deposition of inhaled PM in the airways is determined by the size of the particles, the

respiratory rate, and the anatomy of the respiratory tract. Respiratory particles are deposited in the nasal cavities and the upper airways and mostly eliminated by mucociliary clearance ending up in the gastrointestinal tract.[11, 12] Fine particles go deeper in the bronchial parts of the lung, while UFPs penetrate into the alveoli with the potential to translocate into the blood circulation.[11] Generally, the deposited fraction of particles increases with decreasing size and deeper respiration.

1.1. Health effects consequences air pollution

Environmental air pollutants have a substantial spatial and temporal variation, consequently the effects of these pollutants are difficult to describe on an individual level, but they have a great impact on the population level. Exposure to pollutants such as PM, NO_2 and O_3 has been associated with increased morbidity and mortality [13-16]. These effects have been found in long-term studies, which have followed cohorts of exposed individuals over time, and in short-term studies, which relate day-to-day variations in air pollution and health.[17] Studies have shown that air pollution is a risk factor for cardiovascular and respiratory disease. Daily increases in air pollution levels are related to a higher risk of respiratory symptoms and cardiovascular events including heart failure, angina, myocardial infarction, and death.[18-22] Long-term effect studies of air pollution exposure observed that living in areas with higher levels of air pollution is associated with a slower lung development in children, a higher risk of cardiopulmonary diseases, and an increased mortality.[22-24] These studies indicate that sustained reduction in air pollution exposure should result in improved life expectancy. World Health Organization (WHO) analyzed the effect of combustion-related particulate matter on life expectancy which indicated that current exposure to PM from anthropogenic sources leads to an average loss of 8.6 months of life expectancy in Europe.[4] Furthermore, in the recent update of the Global Burden of Disease, Injuries and Risk Factor study,

air pollution is ranked 5th of a list of the most influential factors influencing health worldwide.[25]

Fetuses, newborns and children are more susceptible to the effects of air pollution exposure compared to adults.[26] In fetuses this is due to their physiologic immaturity and exposure during critical developmental periods (i.e. higher rates of cell proliferation or changing metabolic capabilities).[27] In children this is due to their relative higher ventilation rate and metabolic turnover, as well as by the fact that their organ systems are still in development.[26] Furthermore, their physical behavior, such as spending more time outdoors with a higher physical activity and their closer proximity to traffic emission sources compared to adults, might make them more vulnerable to the adverse effect of airborne pollutants.[26]

Several studies have reported an association between ambient air pollution exposure and neonatal or infant mortality[28], birth weight[29], prematurity[30], and respiratory endpoints, such as the incidence of asthma or impaired lung development.[31] Moreover, in the past two decades, many studies have associated air pollution exposure during pregnancy with important risk factors of impairing fetal growth [32-36]. However, the effects of air pollution exposure on fetal growth are still inconsistent. While some studies suggested that air pollution exposure during pregnancy was significant associated to impaired fetal growth size including smaller head circumference at birth, lower birth weight and shorter birth length [29, 32, 37-39], other studies failed to find such associations [40-43]. Verifying the effects of air pollution on infant growth can help determine the relationships between prenatal exposure to air pollution and adverse health effects later in life, such as respiratory morbidity, cardiovascular disease, childhood obesity, or neurological disorders [44].

The mechanisms by which air pollution exposure can exert these health effects are still unknown, however, oxidative stress and inflammation have been described as important mechanisms.[45]

2. Obesity

WHO defined obesity according to the ranges of body mass index (BMI = weight [kg]/height [m²]. A BMI value within the range of 18.5-24.9 is categorized as normal, 25.0-29.9 as overweight and ≥ 30 as obese.[46] Obesity is a major risk factor for many aging-related diseases – including diabetes, cardiovascular disease, and certain cancers – and it is the leading cause of preventable deaths globally.[46, 47] Obesity can promote these diseases because it is a state of high-systemic oxidative stress and inflammation, characterized by activation of oxidative stress processes and release of inflammatory cytokines.[48]

The prevalence of overweight and obesity is rising worldwide, with the increase in childhood obesity a particular cause for concern.[46] This sharp rise is attributed to the high-calorie diet and the sedentary lifestyle adopted recently by many populations.

Maternal obesity also leads to higher maternal oxidative stress and inflammation status, generating a higher inflammatory and oxidative stress intrauterine environment for the developing fetus. These higher levels of oxidative stress have been proposed to induce metabolic alterations that may act as mechanisms in fetal programming.[49]

3. Age related biomarkers

3.1. Telomeres

Telomeres are noncoding repeated sequences at the end of the chromosomes [5'-(TTAGGG)n-3'] that protect it from degradation and end-to-end fusion to ensure genomic stability and to prevent loss of genetic information.[50] In somatic cells, telomere repeats are lost at each cell division due to the end-replication problem, leading to declines in telomere length with age, and therefore they are considered as a marker of biological aging.[51] During DNA

replication, DNA polymerase is not able to fully replicate the DNA lagging strand, as the last RNA primer cannot be removed an fully replicated. When telomere length reaches a critically short length in one or more chromosomes (also known as the Hayflick limit), end-to-end fusions are formed and genomic instability increases, the cell is signaled to arrest replication and become senescent, with eventual apoptosis.[52-54] To compensate for telomeric DNA loss, telomere structure is regulated by telomerase. Telomerase is involved in the replication of telomeric DNA repeats by adding the telomeric repeat sequence to the end of the chromosomes.[55] Telomerase is a ribonucleoprotein containing a RNA template (TERC) and a reverse transcriptase (TERT) and is mainly active in germ cells, stem cells and immortal cells, and is mainly repressed in somatic cells.[56, 57]

Studies among population show that persons with shorter telomere length in leukocytes have an increased risk for aging-related chronic diseases, such as cancer[58], type 2 diabetes[59], and cardiovascular disease[60]. Although telomere length diminished with age, variations in telomere length between persons of the same chronological age exists, partially reflecting their inherited genetic potential related to replicative senescence.[61-63] This variation may be the result of both genetic and environmental factors.[64]

Oxidative stress and inflammation are major contributors to aging and aging-related chronic diseases such as cardiovascular disease, and also play an important role in accelerated telomere attrition.[65, 66] Telomeres are highly sensitive to oxidative stress, due to their high guanine content and the deficient repair system of single strand breaks.[67] Furthermore, the presence of the unrepaired nucleotides might interface with the replication fork and as such increase telomere shortening.[68] Thus, the key premise of telomere length and attrition as a markers of biological aging and related diseases is that they reflect the cumulative burden of oxidative stress and inflammation occurring over the life course.

3.2. Mitochondria

Mitochondria are intracellular organelles that are essential for cellular energy provision through the production of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. By-products of mitochondrial electron transfer reaction in aerobic cells result in the production of reactive oxygen species (ROS), e.g. superoxide and hydrogen peroxide. Mitochondria are involved in a variety of critical cell functions, including cell proliferation, programmed cell death, signaling transduction, calcium storage and metabolism.[69-71] Each cell contains approximately 200 to 2,000 mitochondria, each carrying 2-10 copies of mitochondrial DNA (mtDNA).[72] The human mtDNA is a double stranded, circular molecule of 16.6 kb and contains 37 genes, encoding 13 proteins that are essential for oxidative phosphorylation and ATP production.[73]

In comparison to the nuclear genome, the mitochondrial genome is more susceptible to oxidative damage. MtDNA is susceptible to ROS generated by the respiratory chain due to its proximity. Therefore, mtDNA is particularly vulnerable to oxidative stress described as an important mechanism by which air pollutants exert their adverse effects.[74] The major differences between human nuclear DNA (nDNA) and mtDNA is that the latter lacks protective histones, chromatine structure, introns and sufficient DNA repair capacity.[73] Consequently, the estimated mutation rate of mtDNA is 5-10 times higher compared to nDNA. Oxidative stress will lead to accumulation of mutations and damage to mitochondrial DNA. Persistent stress can even alter the rate of mtDNA replication and result in a decline in mitochondrial respiratory function. To compensate for this decline, oxidative stress will increase mtDNA content (total amount of mtDNA copies). However, an increase in mitochondria causes excess ROS production and further oxidative damage. This could lead to an accelerated aging process or cell death.[71]

Alterations in mtDNA content is an established marker of mitochondrial damage and function and has been identified as a causal determinant in a

variety of human diseases. Decreased leukocyte mtDNA content has been shown in diabetes type 2[75-77], breast cancer[78, 79], multiple sclerosis[80], renal cell carcinoma[81], and cardiovascular illness[82, 83]. Contrary, increases in mtDNA content have been associated with diseases such as pancreatic cancer[84], lung cancer[85] and intrauterine growth restriction in human placenta[86, 87]. Some mitochondrial disorders can be passed on from mother to child since mtDNA is only transmitter through female germ lines.

4. Main objectives

Many studies have associated air pollution exposure during the most vulnerable stages in life, the in utero period and childhood, but the results are inconsistent. Verifying the effect of air pollution exposure on infant growth can help determine the relationships between prenatal exposure to air pollution and adverse health effects later in life. The mechanisms by which air pollutants exert the adverse health effects are still unknown. Aging begins at the very beginning of life, to accelerate middle-age. Unraveling the complex interplay between exposure to pro-inflammatory risk factors and different biological factors will increase our understanding of DOHaD and the aging phenotype.

We hypothesized that mtDNA content and telomere length, both considered marker of biological aging, are important intermediates or modulating factors between pro-inflammatory risk factors and health outcomes. To this end, I collected data in both newborns and children and addressed the following objectives (Figure 1):

1. To investigate the association between gestational air pollution exposure and placental mtDNA content (chapter 2)
2. To assess the association between gestational air pollution exposure and infant growth (chapter 2 and chapter 3)

3. To evaluate if placental mtDNA content is a possible mediator of the association between prenatal air pollution exposure and infant growth (chapter 2 and chapter 3)
4. To investigate whether leukocyte telomere length at 8 years of age is related to early life air pollution exposure (chapter 4)
5. To evaluate the association between indicators of maternal and childhood obesity and adiposity and telomere length measured in children aged 8 years (chapter 5)

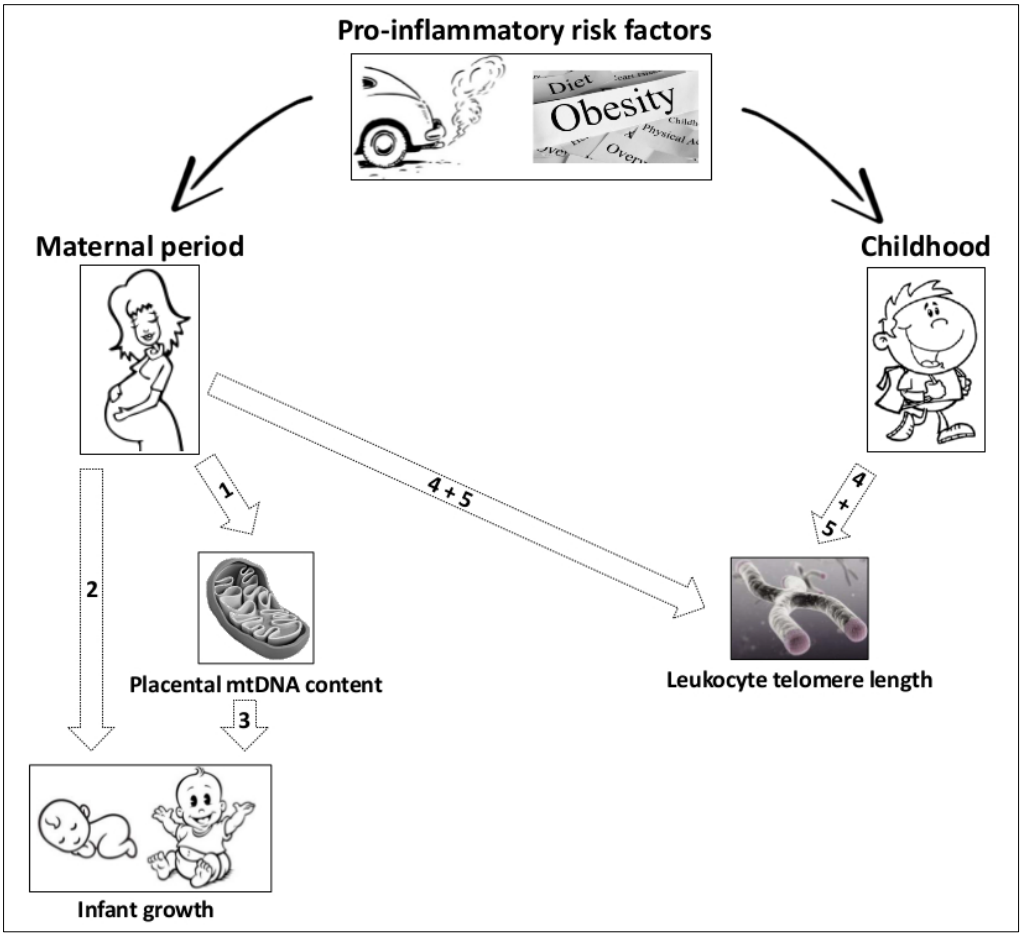

Figure 1. Schematic overview of the objectives of this doctoral dissertation

Chapter 2.

Prenatal Ambient Air Pollution, Placental Mitochondrial DNA Content, and Birth Weight in the INMA (Spain) and ENVIRONAGE (Belgium) Birth Cohorts

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Chapter 3.

Prenatal ambient air pollution exposure, infant growth and placental mitochondrial DNA content in the INMA birth cohort

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Chapter 4.

Early life traffic-related air pollution exposure predicts telomere length in 8 year-olds

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Submitted

Abstract

Background Telomere length is a molecular marker of aging. Here we investigated whether leukocyte telomere length (LTL) at 8 years of age was associated with early life exposure to residential air pollution.

Methods In a multi-centre European birth cohort study HELIX (Human Early-Life Exposome) (n=1396), we estimated prenatal and postnatal exposure to nitrogen dioxide (NO_2), particulate matter with aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$), and proximity to major roads. Average relative LTL was measured using real-time polymerase chain reaction (qPCR). Effect estimates of the association between LTL and prenatal, postnatal and proximity to major roads were calculated using multiple linear mixed models with a random cohort effect and adjusted for relevant covariates.

Results LTL was inversely associated with prenatal and postnatal NO_2 and $PM_{2.5}$ exposures levels. Childhood leukocyte telomeres for each SD increase in prenatal NO_2 was associated with a -1.5% (95% CI -2.8, -0.2) change in LTL. However, each SD increment in prenatal $PM_{2.5}$ was non-significantly associated with LTL (-0.7%; 95% CI: -2.0, 0.6). For each SD increment in postnatal NO_2 and $PM_{2.5}$ exposure LTL shortened with -1.6% (95% CI: -2.9, -0.4) and -1.4% (95% CI: -2.9, 0.1), respectively. Each doubling in residential distance to nearest major road during childhood was associated with 1.6% (95% CI: 0.02, 3.1) longer LTL.

Conclusion In conclusion, healthy air both during prenatal and postnatal life is associated with longer telomere length in children. These results suggest that reductions in traffic related air pollution may promote molecular longevity from early life onwards.

Introduction

In the recent update of the Global Burden of Disease, Injuries and Risk Factor study, air pollution is ranked 5th of a list of the most influential factors affecting health worldwide [25]. Hypothesis are that oxidative stress and inflammation are important underlying mechanisms through which air pollutants could cause adverse health outcomes [131].

Telomeres are complexes of tandem repeats of DNA (5'-TTAGGG-3'), sited at the termini of the chromosomes. Telomeres have a significant function in maintaining the integrity of chromosomes and the stability of the genome, and prevent end-to-end chromosomal fusions [50]. Since DNA polymerase is unable to fully replicate the 3' end of the DNA strand, telomeres shorten with each cell division. Consequently, telomere length is considered a biomarker of biological aging and shorter telomeres have been associated with age-related diseases such as cardiovascular disease [60, 152, 153], type 2 diabetes [154] and increased mortality [155-157]. Furthermore, it is believed that the natural erosion of telomeres is accelerated through oxidative stress and inflammation [158].

According to the Developmental Origins of Health and Disease (DOHaD) small changes in the early life environment shape the future probability of the development of age-related diseases [1, 88]. The rate of telomere attrition is greatest in young children [159] and the telomere length decline then continues at a slower rate throughout adulthood [160] Consequently, telomere loss in childhood is a potential important factor leading to the ultimate telomere length in adults. Environmental factors might have the greatest effect in childhood when the high telomere attrition is occurring.

Air pollution exposures may contribute to the aging-phenotype and telomere length may play a mechanistic role in linking air pollution to age-related diseases. It is thus important to study the link between early life air pollution exposure and telomere length in childhood to gain insights in the etiology of age-related diseases. Here, we assessed, within a multi-centre birth cohort study in six European countries with a wide range of exposures, the

association between prenatal and postnatal exposure to air pollution as exemplified by residential nitrogen dioxide (NO_2), particulate matter ($PM_{2.5}$), and residential proximity to major road, and leukocyte telomere length (LTL) in 8 year old children.

Methods

Study population and data collection

The Human Early-Life Exposome (HELIX) study is a collaborative project across six established and ongoing longitudinal population-based birth cohort studies in Europe: the Born in Bradford (BiB) study in the UK [161], the Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) study in France [162], the INfancia y Medio Ambiente (INMA) cohort in Spain [163], the Kaunus cohort (KANC) in Lithuania [164], the Norwegian Mother and Child Cohort Study (MoBa) [165] and the RHEA Mother Child Cohort study in Crete, Greece [166]. The study population for the entire HELIX cohort includes 31,472 women who had singleton deliveries between 1999 and 2010, and for whom exposure to ambient air pollution during pregnancy had been estimated as part of the ESCAPE project [29]. Local ethical committees approved the studies that were conducted according to the guidelines laid down in the Declaration of Helsinki. All participating women provided informed written consent. The analysis of this paper made use of the HELIX subcohort that includes mother-child pairs who were fully characterised for a broad suite of environmental exposures, to be clinically examined, and to have biological samples collected. A new follow-up visit was organised for these mother-child pairs. Subcohort subjects were recruited from within the entire cohorts such that there were approximately 200 mother-child pairs from each of the 6 cohorts. Subcohort recruitment in the EDEN cohort was restricted to the Poitiers area and in the INMA cohort to the city of Sabadell.

Detailed information on maternal age at birth, maternal education, maternal marital status, smoking status during pregnancy, parity, and maternal ethnicity

from each study participant was obtained by each cohort during pregnancy or at birth by questionnaire or medical records. The level of maternal education reported by the participant was used as the primary indicator of SES and categorized according to the International Standard Classification of Education (ISCED) as three levels: "low" (less than primary, primary, and lower secondary education, ISCED 2011 levels 0-2); "middle (upper secondary and post-secondary non-tertiary education, ISCED 2011 level 3 and 4); high" (tertiary education, ISCED 2011 levels 5-8). Maternal smoking status was categorized as "no active smoking during pregnancy" and "active smoking during pregnancy". Child ethnicity was defined for all cohorts and subdivided in 7 different groups (African, Asian, White European, Mixed native-American, South-Asian, White- not European, or others). Perinatal parameters such as birth date, and newborn sex were obtained at birth.

Blood collection and DNA extraction

DNA was obtained from buffy coat collected in EDTA tubes. 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 DNA integrity was tested with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies).

Average relative telomere length measurement

Average relative telomere length was measured by a modified qPCR protocol as described previously [167]. Telomere and single copy-gene reaction mixture and PCR cycles used can be found in Martens et al [168]. 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 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.

Exposure assessment

We assessed both prenatal and postnatal air pollution exposure at the residential address during pregnancy and follow-up). Air pollutants used in this study included nitrogen dioxide (NO_2), and particulate matter with an aerodynamic diameter of less than 2.5 µm ($PM_{2.5}$). These air pollutants were estimated using land use regression (LUR) or dispersion models, temporally adjusted to measurements made in local background monitoring stations and averaged over trimester 1, trimester 2, trimester 2 and the whole pregnancy period. For most cohorts, we used site-specific LUR models developed in the context of the ESCAPE project [169, 170]. In EDEN, dispersion models were used to assess the NO_2 exposure [171] and the ESCAPE European-wide LUR model was applied for $PM_{2.5}$, corrected for local background monitoring data [172]. In BiB, $PM_{2.5}$ assessment was made based on the ESCAPE LUR model developed in the Thames Valley region of the UK and adjusted for background PM levels from monitoring stations in Bradford [32]. Additionally, we collected information on the traffic assessed as distance to nearest road (m). Postnatal air pollution included annual NO_2, and $PM_{2.5}$, assessed for the year before the telomere length measurements through site-specific ESCAPE LUR models for all cohorts except EDEN. In EDEN, a local dispersion model was used to assess the NO_2 exposure. Additionally, we assessed traffic levels as distance to nearest road (m) at the child's home residence.

The software used to make the spatial analysis were ArcGIS platform (ESRI ArcMap TM 10.0, ArcGIS Desktop 10 Service Pack 4) and spatialite v.4.11.

Statistical analysis

We performed multiple imputation using chained equations to account for missing values of air pollution and potential confounding variables, 20 datasets were generated and pooled for analyses (Supplemental Materials). LTL showed a skewed distribution and was therefore log_{10} transformed to achieve a normal distribution. Generalized additive models (GAMs) were used to assess the linearity of the associations between leukocyte telomere length and pre- and postnatal air pollution exposure. Multiple linear mixed models with a random cohort effect were applied to test the association between leukocyte telomere length at 8 years of age (ranged 5.4 – 12.0 years) and traffic and air pollution exposures and LTL at age 8 years. All the used models were adjusted for a priori chosen covariates including child's age, sex, qPCR batch, maternal age, maternal education, maternal smoking status during pregnancy, child ethnicity, child BMI, parental smoking at 8 year, and blood cell type proportions.

In a first step of the analysis, we studied leukocyte telomere length by medians of the distributions of the exposure variables considered separately. In the next step, air pollutants were treated as continuous variables and were scaled to a standard deviation (SD) difference in level for testing associations with leukocyte telomere length. Distance to nearest road was log10 transformed to assure normality. A multiple pollutant models considering simultaneously $PM_{2.5}$ and NO_2 levels in each exposure window were assessed. Finally, we used models that mutually adjusted for prenatal and postnatal exposure to assess which period had the largest effect on LTL.

To test whether the results were robust we ran different sensitivity analyses in which we tested the sex interaction between air pollution exposure and sex on LTL in children by including its interaction term in the full models. The sensitivity of the findings was also examined by removing one study at the time form the analysis and recalculating the estimates.

Analyses were performed using the SAS 9.3 statistical software (SAS Institute Inc. Cary, NC, USA).

Results

Characteristics of the study population

Table 1 describes the general characteristics of the study population (n = 1396). 643 (46.1%) of the children were girls, were mainly White European (87.4%), and had a mean (SD) age of 8 (1.5) years. Mean (SD) maternal age at delivery was 30.5 (4.9) years. 643 (46.1%) of the mothers were highly educated, 635 (45.5%) of the mothers were primiparous and 229 (13.4%) of the mothers actively smoked during pregnancy. The characteristics for the individual cohorts are presented in the Supplemental Materials (Table S1).

Table 2 displays the average outdoor prenatal and postnatal air pollution exposures. Average (25-75th percentile) mean prenatal exposure was 25.0 (14.8-32.9) µg/m³, and 15.1 (13.5-16.9) µg/m³, for NO2, and PM2.5 respectively. Average (25-75th percentile) mean postnatal exposure was 23.1 (11.9-32.2) µg/m³, and 13.2 (11.0-15.0) µg/m³, for NO2, and PM2.5 respectively. Prenatal and postnatal NO2 were highly correlated, whereas a similar analysis for PM2.5 showed a moderate correlation (Table 2). The exposure characteristics for the individual cohorts are presented in the Supplemental Materials (Table S2).

Table 1. General characteristics of the complete case study population (n=1396)

Children	**Mean (SD) or n (%)**
Sex	
Girls	643 (46.1)
Boys	753 (53.9)
Ethnicity	
African	12 (0.9)
Asian	21 (1.5)
White European	1223 (87.4)
Mixed native_American	13 (0.9)
Other	22 (1.6)
South-Asian	79 (5.7)
White not European	26 (1.9)
Cohort	
INMA	428 (30.6)
MOBA	213 (15.3)
BIB	205 (14.7)
RHEA	202 (14.5)
KANC	199 (14.3)
EDEN	149 (10.6)
Age at telomere length assessment, years	8.0 (1.5)
Relative telomere length	1.0 (0.9 – 1.1)
zBMI	0.48 (1.2)
Mothers	**Mean (SD) or n (%)**
Age at delivery, years	30.5 (4.9)
Missing	15 (1.1)
Education	
Low	219 (15.8)
Middle	480 (34.5)
High	643 (46.2)
Missing	54 (3.5)
Active smoking during pregnancy	
Yes	1121 (83.3)
No	229 (13.4)
Missing	46 (3.30)
Parity	
1	635 (45.5)
2	498 (35.7)
≥3	228 (16.3)
Missing	35 (2.5)
Parental smoking at 8 years	
Neither	827 (59.3)
One	394 (28.2)
Both	156 (11.2)
Missing	19 (1.3)

Continuous covariates expressed by mean and standard deviation (SD) or geometric mean and 25–75th percentile; categorical covariates described by number and frequencies (%).

Table 2. Exposure characteristics of the complete case study population (µg/m³)

			Percentiles					Correlation[a]	
	n	Mean ± SD	5th	25th	50th	75th	95th	Prenatal	Postnatal
NO_2									
Prenatal	1237	25.0 ± 13.9	9.6	14.8	20.4	32.9	51.4	1	
Postnatal	1366	23.1 ± 12.2	7.3	11.9	23.3	32.2	42.2	0.74*	1
$PM_{2.5}$									
Prenatal	1307	15.1 ± 2.6	10.7	13.5	15.0	16.9	19.6	1	
Postnatal	1366	13.2 ± 3.3	7.3	11.0	13.3	15.0	19.1	0.48*	1

Continuous variables expressed by mean and standard deviation ± SD
[a]Spearman correlation coefficient between prenatal and postnatal exposure
*P-value < 0.0001

Association between leukocyte telomere length and maternal and child characteristics

We observed shorter LTL in boys compared to girls (0.98 vs 1.02, p<0.0001), while LTL was within our narrow age range not significantly correlated with child´s age (r = -0.038, P= 0.15). Shorter LTL in children were associated with higher child BMI (r = -0.073; P = 0.007). Childs telomere lengths was positively associated with maternal age (r = 0.09, P= 0.0006).

Association between leukocyte telomere length at age of 8 years and prenatal and postnatal air pollution

Figure 1 shows the GAMs of the different associations. The GAMs did not show non-linearity (p-gain > 0.05). Table 3 presents categorical analysis for comparing high (above median) versus low pre- or postnatal exposure (below median) in association with childhood LTL. Prenatal NO_2 above the median were associated with 3.0% (95% CI: -5.2, -0.8) shorter telomeres compared with the exposure below the median. Additionally, postnatal $PM_{2.5}$ exposures were associated with 3.0% (95% CI: -5.3, -0.6) shorter telomeres in the group exposed above 13.3 µg/m³ compared with the group below this value (Table

3). We did not observed a significant association between LTL and prenatal $PM_{2.5}$ or postnatal NO_2 exposure.

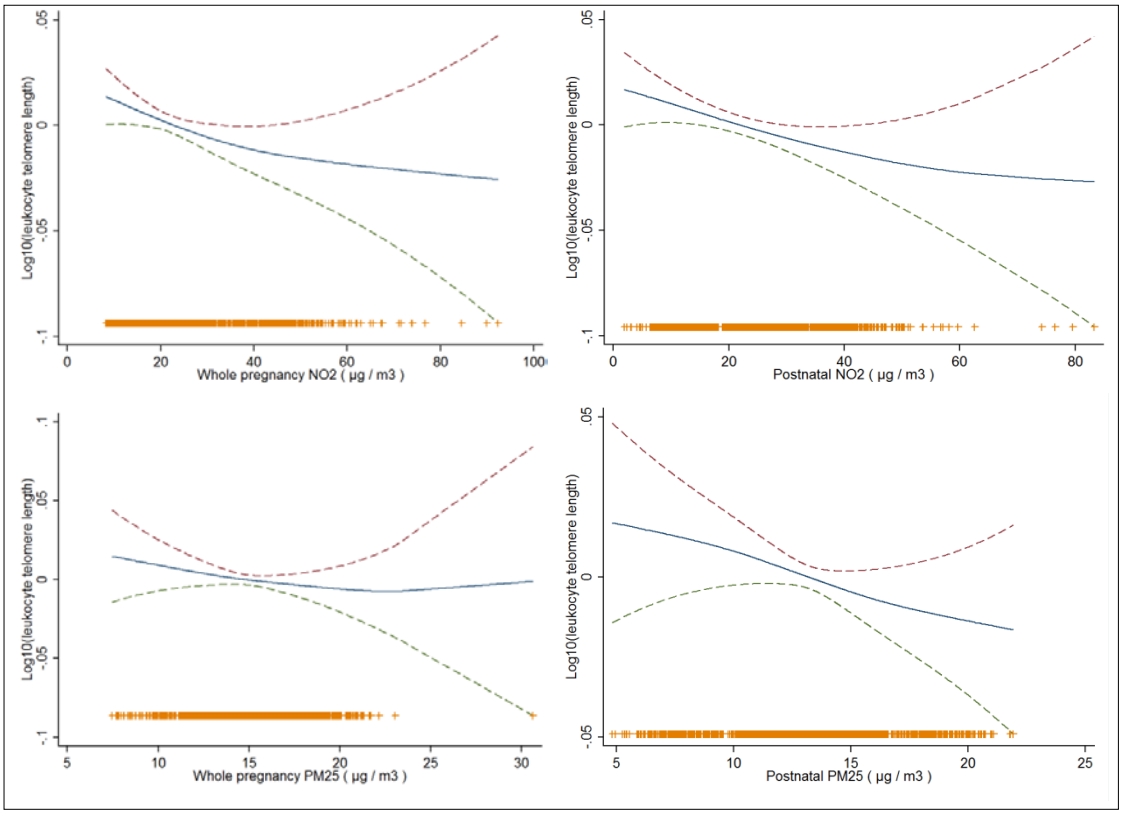

Figure 1. GAM models show the linear relation between (A) Prenatal NO_2 exposure (µg/m3) during the entire pregnancy and child leukocyte telomere length, (B) Postnatal NO_2 exposure (µg/m3) and child leukocyte telomere length, and (C) $PM_{2,5}$ exposure (µg/m3) during the entire pregnancy and child leukocyte telomere length (D) Postnatal $PM_{2,5}$ exposure (µg/m3) during the entire pregnancy and child leukocyte telomere. Models were adjusted for child's age, sex, qPCR batch, maternal age, maternal education, maternal smoking status during pregnancy, child ethnicity, child BMI, parental smoking at 8 year, and blood cell type proportions

Table 3. Leukocyte telomere length in association with categorized pre- and postnatal ambient air pollution.

	% Change (95% CI)	p-value
Prenatal		
NO_2 < 20.5 µg/m³	Ref	
≥ 20.5 µg/m³	-3.0 (-5.2 to -0.8)	0.008
$PM_{2.5}$ < 15.0 µg/m³	Ref	
≥ 15.0 µg/m³	-0.9 (-3.1 to 1.4)	0.43
Distance to nearest road > 150 m	Ref	
≤ 150 m	-1.7 (-4.6 to 1.3)	0.26
Postnatal		
NO_2 < 23.5 µg/m³)	Ref	
≥ 23.5 µg/m³	-1.8 (-4.2 to 0.67)	0.15
$PM_{2.5}$ < 13.3 µg/m³	Ref	
≥ 13.3 µg/m³)	-3.0 (-5.3 to -0.62)	0.01
Distance to nearest road > 150 m	Ref	
≤ 150 m)	-1.9 (-4.0 to 0.31)	0.09

Models were adjusted for child's age, sex, qPCR batch, maternal age, maternal education, maternal smoking status during pregnancy, child ethnicity, child BMI, parental smoking at 8 year, and blood cell type proportions.

Figure 2 presents the estimates of the continuous association between a SD increment in prenatal (different trimesters and whole pregnancy) and postnatal air pollution exposure and LTL in 8 year-old children. Leukocyte telomere length was statistically significantly and inversely associated with NO_2 exposure during pregnancy (-1.5%; 95% CI: -2.8, -0.2) but not with $PM_{2.5}$ exposure (-0.7%; 95% CI: -2.0, 0.6). Similarly, a SD increment in postnatal NO_2 was associated with statistically significant shorter leukocyte telomere length (-1.6%; 95% CI: -2.9, -0.4) at age 8 years. Furthermore, postnatal $PM_{2.5}$ was inversely (-1.4%; 95% CI: -2.9, 0.1) associated with telomere length at age 8 years, although this was only borderline statistically significant. Doubling of the residential proximity to nearest road during pregnancy was not significantly associated with childhood telomere length (0.2%; 95% CI: -1.3, 1.6), whereas, a doubling in residential proximity to nearest road in postnatal life was associated with a significant longer childhood telomere length (1.6%; 95% CI: 0.02, 3.1) (Figure 2).

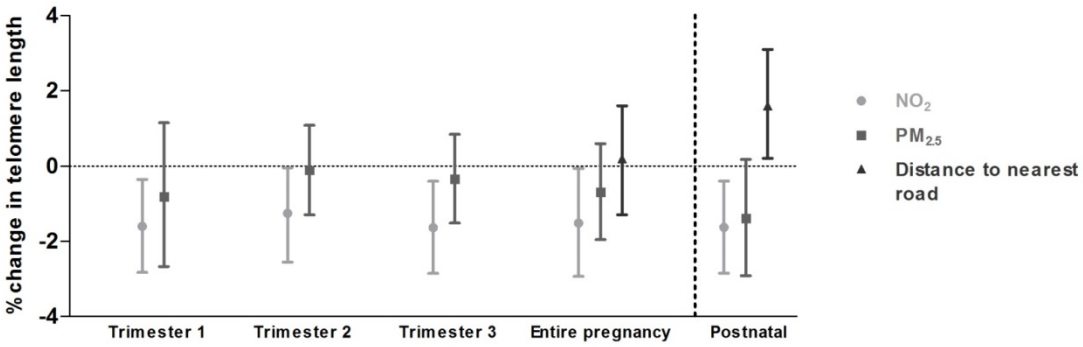

Figure 2. The association leukocyte telomere length between prenatal (trimester specific and whole pregnancy)/postnatal traffic-related air pollution/distance to nearest road and in 8-year old children; Effect size (% change with 95%CI) was estimated for each SD increment in exposure to NO_2, and $PM_{2.5}$ and doubling in distance to nearest road; Models were adjusted for child's age, sex, qPCR batch, maternal age, maternal education, maternal smoking status during pregnancy, child ethnicity, child BMI, parental smoking at 8 year, and blood cell type proportions

Ambient air pollutants (NO_2, and $PM_{2.5}$) were weakly correlated with each other ((r = 0.20, p < 0.0001; r = 0.15, p < 0.0001) for prenatal and postnatal exposure, respectively). Therefore, multi-pollutant models which included both NO_2, and $PM_{2.5}$ did not alter interpretation of the results (Table 4). Prenatal and postnatal NO_2 were highly correlated, whereas a similar analysis for $PM_{2.5}$ showed a moderate correlation (Table 2), therefore it is difficult to distinguish the effects of prenatal and postnatal air pollutants. However, results from models that mutually adjusted for prenatal and postnatal exposure suggest that the effects on telomere length were due to postnatal rather than prenatal exposure (Table 5).

Table 4. Association between leukocyte telomere length and traffic-related air pollution exposure and in a multi-pollutant model

	% Change (95% CI)	p-value
Prenatal exposure[a]		
NO_2	-1.9 (-3.3 to -0.6)	0.006
$PM_{2.5}$	-0.6 (-1.8 to 0.6)	0.3
Postnatal exposure[b]		
NO_2	-1.5 (-2.7 to -0.4)	0.01
$PM_{2.5}$	-1.5 (-3.2 to 0.2)	0.1

Effect size was estimated as a % change in LTL for each SD increment in ambient air pollution exposure; SD prenatal NO_2 = 13.9 μg/m^3, SD postnatal NO_2 = 12.2 μg/m^3, SD prenatal $PM_{2.5}$ = 2.6 μg/m^3, SD postnatal $PM_{2.5}$ = 3.3 μg/m^3

[a]Model included both average entire pregnancy NO_2 and $PM_{2.5}$ exposure terms

[b]Model included both average year NO_2 and $PM_{2.5}$ exposure terms prior to LTL assessment

Models were adjusted for child's age, sex, qPCR batch, maternal age, maternal education, maternal smoking status during pregnancy, child ethnicity, child BMI, parental smoking at 8 year, and blood cell type proportions

Table 5. Association between traffic-related air pollution exposure and telomere length in a multi time window model

	% Change	95% CI	p-value
NO_2[a]			
Whole pregnancy	-0.69	-2.65,1.32	0.5
Postnatal	-1.16	-2.96,0.67	0.21
$PM_{2.5}$[b]			
Whole pregnancy	0.29	-1.35,1.95	0.73
Postnatal	-1.61	-3.59,0.42	0.12

Effect size was estimated as a % change in LTL for each SD increment in ambient air pollution exposure; SD prenatal NO_2 = 13.9 μg/m^3, SD postnatal NO_2 = 12.2 μg/m^3, SD prenatal $PM_{2.5}$ = 2.6 μg/m^3, SD postnatal $PM_{2.5}$ = 3.3 μg/m^3

[a]Model included both average entire pregnancy NO_2 and postnatal NO_2 terms

[b]Model included both average entire pregnancy $PM_{2.5}$ and postnatal $PM_{2.5}$ terms

Models were adjusted for child's age, sex, qPCR batch, maternal age, maternal education, maternal smoking status during pregnancy, child ethnicity, child BMI, parental smoking at 8 year, and blood cell type proportions

Sensitivity analyses

Interaction tests showed that the interaction of air pollution exposure with sex was not significant for the different models (data not shown). The sensitivity of the significant findings was further examined by removing one cohort at the time from the analyses and recalculate the % change in telomere length. The % change in telomere length for each increment in prenatal NO_2 exposure increased to -3.4% when INMA was excluded and to -1.7% when RHEA was excluded, while excluding MOBA lead to a drop in % change in telomere length off -1.1%. Excluding BIB, KANC or EDEN did not change our reported % changes in telomere length. Additionally, the % change in telomere length for each increment in postnatal NO_2 exposure increased to -2.1% when INMA was excluded and to -1.8% when KANC was excluded, while excluding BIB, RHEA or EDEN lead to a drop in % change in telomere length of -1.2%, -0.9% and -1.4%, respectively. Excluding MOBA did not alter our reported % changes in telomere length.

Discussion

The present study, including 6 populations across Europe, is so far the largest study of air pollution exposure and LTL in children. Here we showed that prenatal (entire pregnancy) and postnatal (1 year prior blood collection) traffic related air pollution were associated with shorter leukocyte telomeres in children. For each SD increment in prenatal NO_2 (13.9 ug/m3) LTL were -1.5% shorter in children. Additionally, for each SD increment (12.2 ug/m3, 3.3 ug/m3) in postnatal NO_2 and $PM_{2.5}$ exposure LTL was -1.6% and -1.4% shorter, respectively.

Recent epidemiological studies showed an association between air pollution and adverse health outcomes, including cardiovascular and respiratory diseases [14, 15, 173, 174]. Long-term exposure to traffic-related air pollution is associated with premature mortality. A study from Norway reported an increase of 18% in male all-cause mortality based on a comparison of the lowest to the

highest quartile of exposure in ambient residential nitrogen oxides (NO_x) [175]. A Canadian study [176] showed that persons with a close residential proximity to major road (buffers of 50 m around major urban roads and 100 m from highways) had mortality rate advancements of 2.5 years and a significant increase in all-cause mortality of 18%. The European ESCAPE analysis found a significantly increased hazard ratio of 1.07 (95% CI: 1.02, 1.13) for natural-cause mortality per 5 μg/m^3 increment in $PM_{2.5}$ exposure [173]. Conversely, improvements in air pollution parallel increases in the US population life-expectancy which could not be attributed to demographic or social economical changes [177].

The biological mechanisms by which air pollutants may cause adverse health outcomes are not completely understood, but oxidative stress and inflammation are thought to be of importance. The ability of oxidative stress to damage nucleic acids provides a potential mechanism by which oxidative stress could interfere with telomere DNA [178]. It is assumable that telomeres are a sensitive target for ROS-induced damage, as telomeres contain a high amount of ROS sensitive guanine bases[179]. ROS can induce DNA breakage, and single strand breaks in telomeric DNA is ineffectively repaired, leading to increased telomere shortening [180].

We found a significant inverse association between prenatal and postnatal air pollution exposure and telomere length at 8 year of age. Our findings of prenatal exposure and LTL in children are in line with studies in newborns [181, 182]. In the East Flanders Prospective Twin Survey, maternal residential proximity to a major road was associated with placental telomere length: a doubling in the distance to the nearest major road was associated with 5.32% longer placental telomere length at birth [181]. In 641 newborns of the ENVIR*ON*AGE birth cohort, cord blood and placental telomere length were significantly inversely associated with $PM_{2.5}$ exposure during mid-gestation with approximately 8.8% and 13.2% shorter cord blood and placental telomere length at birth for each 5 μg/m³ increase in residential $PM_{2.5}$ exposure, respectively [182]. We found that the association between telomere length and

exposure to air pollution is persistent into childhood and in addition postnatal air pollution exposure adds upon this effect. In contrast to our current study and previous studies [138, 183-185], a study of school children in London reported that annual air pollution exposure was associated with longer telomeres in saliva, DNA coming from a mixture of different cell types [186]. The authors suggested that these increases in telomere length may be due to the effect on telomere associated proteins, telomerase activation, or clonal expansion of less mature leukocytes [187, 188] or it could also be due to difficulties in measuring telomeres in the saliva matrix.

How do our results in childhood compare to the evidence in adults? The Normative Aging Study found an inverse association between long-term exposure to ambient black carbon (BC) and telomere length in adulthood (-7.6% for each 0.25 μg/m³ increment in BC; 95% CI: -12.8, -2.1)[183]. A cross-sectional study on traffic officers and indoor office workers found that traffic officers (LTL = 1.02; 95% CI: 0.96-1.09) had shorter leukocyte telomeres than did office workers (LTL = 1.22; 95% CI = 1.13-1.31), suggesting that long-term exposure to traffic related air pollution may shorten telomere length [184]. Furthermore, a study in the KORA F4 cohort found that telomere length was inversely associated with black carbon in men (β = −0.28; 95% CI = −0.47, −0.1)[189].

Traffic related air pollution in the early life environment as exemplified by residential ambient NO_2 exposure both prenatal and during childhood may increase the risk for chronic diseases in adulthood. Indeed, although telomeres of children are long compared with adults, shortening due to early life exposure to air pollution may decrease the buffer capacity to cope with inflammation and oxidative stress later in life and therefore it is reasonable to assume that it might lead to faster shortening of critical telomere length at older age. We were not able to estimate the effects of our decline based on absolute values of telomere length, since we used a real-time PCR method that cannot provide these absolute values. Nevertheless, an estimation can be based on available

data in the literature. In young adulthood telomere length are on average 8 kb [190] and the annual telomere loss in adult leukocytes is between 32.2 and 45.5 bp [191]. Prenatal NO_2 exposures by the median (20.5 µg/m³) was associated with a 3.0% (95% CI: 5.2, -0.8) shorter telomeres in the group exposed above 20.5 µg/m³ compared with the group below this value. This reduction of 3.0% corresponds to a reduction of 240 bp indicating that this effect-size of 3.0% shortening is equivalent to a loss of 5.3 to 7.4 years (based on telomere attritions of 32.2-45.5 bp per year). Taken together this illustrates the public health significance of our findings, as based on telomeric year equivalence in adulthood, children from mothers exposed above the median during pregnancy were biologically (in terms of telomere shortening) approximately 6 years older.

Our study needs to be interpreted within the context of its potential limitations. Firstly, the traditional method to determine telomere length is telomere restriction fragment (TRF) analysis. In this study we used a real-time PCR method which has, in general, a higher assay variability compared to the TRF method [192, 193]. However, an inter-laboratory comparison of our method showed that the coefficient of variation was less than 7%. Secondly, the assessment of telomere length at 8 year of age represents only a snapshot in childhood. We were not able to evaluate telomere dynamics throughout the entire pregnancy and the childhood period. Thirdly, paternal age exerts a considerable effect on child telomere length [194], however, this data was not available in our cohorts. Fourthly, we only looked at 2 exposure periods during the child's life including exposure *in utero* and the recent postnatal life (one year before assessment of telomere length). However, the exposures in these periods were highly correlated and therefore difficult to distinguish. Finally, our results are based on exposure at the home address, and potential misclassification may be present because we could not account for other exposure sources that contribute to personal exposure, such as exposure during commute, at work or school, and elsewhere.

In conclusion, in a large multicenter European cohort we showed that traffic related air pollution exposure in early life is associated with childhood telomere

length . Our evidence of biomolecular harm helps to elucidate causal pathways between air pollution and later adverse health outcomes and suggests that reduction of traffic related air pollution levels may promote molecular longevity from early life onwards.

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Supplemental material

Supplemental Table S1. General characteristics of the complete case study population stratified by cohort

	INMA (n = 428)	MOBA (n = 213)	BIB (n = 205)	RHEA (n = 199)	KANC (n = 202)	EDEN (n = 149)
Children						
Sex						
Girls	206 (48.13)	98 (46.0)	93 (45.37)	89 (44.72)	92 (45.54)	65 (43.6)
Boys	222 (51.87)	115 (54.0)	112 (54.63)	110 (55.28)	110 (54.46)	84 (56.4)
Ethnicity						
African	5 (1.17)	0 (0.0)	7 (3.41)	0 (0.0)	0 (0.0)	0 (0.0)
Asian	2 (0.47)	6 (2.9)	13 (6.34)	0 (0.0)	0 (0.0)	0 (0.0)
White European	380 (88.32)	204 (95.7)	89 (43.41)	199 (100.0)	202 (100.0)	149 (0.0)
Mixed native American	11 (2.57)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other	4 (0.93)	1 (0.4)	17 (8.29)	0 (0.0)	0 (0.0)	0 (0.0)
South-Asian	0 (0.0)	0 (0.0)	79 (38.54)	0 (0.0)	0 (0.0)	0 (0.0)
White not European	26 (6.07)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Gestational age, weeks	39.9 ± 1.4	40.1 ± 1.7	39.7 ± 1.8	38.4 ± 1.4	39.4 ± 1.3	39.8 ± 1.7
Child age, years	9.02 ± 0.65	8.5 ± 0.5	6.6 ± 0.2	6.5 ± 0.3	6.5 ± 0.5	10.8 ± 0.6
Mothers						
Age at delivery	31.5 ± 4.2	32.8 ± 3.7	28.6 ± 5.8	30.9 ± 4.8	28.57 ± 5.0	30.7 ± 5.0
Missings	1 (0.2)	6 (2.8)	1 (0.5)	2 (1.0)	2 (1.0)	0 (0.0)
Education						
Low	99 (23.1)	0 (0.0)	88 (42.9)	9 (4.5)	12 (5.9)	11 (7.4)
Middle	174 (40.7)	41 (19.2)	31 (15.1)	111 (55.8)	69 (34.2)	55 (36.9)
High	141 (32.9)	164 (77.0)	64 (31.2)	79 (39.7)	116 (57.4)	83 (55.7)
Missings	14 (3.3)	8 (3.8)	22 (10.7)	2 (1.0)	5 (2.5)	3 (2.0)
Active smoking during pregnancy						
Yes	109 (225.46)	9 (4.4)	25 (12.2)	43 (21.6)	13 (6.44)	31 (20.8)
No	311 (72.66)	198 (91.6)	157 (76.6)	156 (78.4)	184 (91.09)	118 (79.2)
Missings	8 (1.87)	9 (4.4)	23 (11.2)	1 (0.5)	5 (2.5)	0 (0.0)
Parity						
1	230 (53.7)	93 (43.7))	83 (40.5)	74 (37.2)	84 (41.6)	71 (47.7)
2	165 (38.6)	86 (40.4)	52 (25.4)	85 (42.7)	59 (29.2)	51 (34.2)
≥3	28 (6.5)	28 (13.1)	56 (27.3)	35 (17.6)	54 (26.7)	27 (18.1)
Missings	5 (1.2)	6 (2.8)	14 (6.8)	5 (2.5)	5 (2.5)	0 (0.0)

Continuous covariates expressed by mean and standard deviation ± SD; categorical covariates described by number and frequencies (%).

Supplemental Table S2. Exposure characteristics of the complete case study population stratified by cohort

				Percentile				
	n	**Mean**	**SD**	**5th**	**25th**	**50th**	**75th**	**95th**
INMA								
NO_2 Prenatal	351	43.23	11.17	24.55	36.08	43.24	49.07	60.83
NO_2 Postnatal	401	33.04	11.81	11.74	26.83	35.4	40.69	50.18
$PM_{2.5}$ Prenatal	351	15.08	1.72	12.3	14.1	14.97	15.98	17.86
$PM_{2.5}$ Postnatal	401	13.31	1.72	10.47	12.66	13.3	13.88	15.7
MOBA								
NO_2 Prenatal	206	20.51	7.67	11.17	14.49	18.52	25.24	36.31
NO_2 Postnatal	207	26.2	5.41	19.35	22.72	25.44	29.59	33.6
$PM_{2.5}$ Prenatal	207	12.06	2.22	8.13	10.47	12.12	13.53	15.94
$PM_{2.5}$ Postnatal	207	8.12	1.61	5.95	7.06	7.77	9.06	11.13
BIB								
NO_2 Prenatal	205	20.79	3.43	15.66	18.43	20.61	23.12	26.71
NO_2 Postnatal	205	31.6	3.93	26.68	28.61	31.29	33.67	38.11
$PM_{2.5}$ Prenatal	205	14.37	1.78	11.49	13.28	14.18	15.48	17.5
$PM_{2.5}$ Postnatal	205	14.39	1.2	12.66	13.58	14.23	15.12	16.44
RHEA								
NO_2 Prenatal	199	12.14	4.21	8.34	9.28	11.19	12.8	21.83
NO_2 Postnatal	199	10.99	3.47	7.66	6.87	10.09	12.05	18.72
$PM_{2.5}$ Prenatal	199	14.49	1.24	12.95	12.95	14.39	15.26	16.99
$PM_{2.5}$ Postnatal	199	14.09	1.86	11.71	12.83	13.63	15.12	17.47
KANC								
NO_2 Prenatal	195	18.53	3.74	13.42	15.94	17.83	20.67	24.79
NO_2 Postnatal	194	13.99	2.51	10.05	12.51	13.99	15.21	17.8
$PM_{2.5}$ Prenatal	195	17.61	2.44	13.49	15.78	17.98	19.09	20.93
$PM_{2.5}$ Postnatal	194	18.29	1.6	15.28	17.58	18.28	19.34	20.68
KANC								
NO_2 Prenatal	80	15.06	5.16	9.94	11.65	13.38	17.32	24.28
NO_2 Postnatal	148	8.3	1.96	6.44	6.74	7.71	9.13	12.56
$PM_{2.5}$ Prenatal	149	18.09	1.54	15.52	17.12	18.09	18.99	20.87
$PM_{2.5}$ Postnatal	148	10.68	0.44	10.17	10.39	10.62	10.87	11.38

Continuous variables expressed by mean and standard deviation (SD)

Chapter 5.

Obesity indicators are associated with shorter telomeres in 8 year old children

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In preparation

Abstract

Introduction Telomere length is considered a biomarker of biological aging. Shorter telomeres and obesity have both been associated with age-related diseases such as cardiovascular disease and type 2 diabetes. Moreover, in recent years, obesity has been associated with shorter telomeres in adults but information is lacking in children. In this study we evaluated the association between various indices of obesity with telomere length in childhood.

Methods In 1,396 children of the multi-centre European birth cohort study HELIX, anthropometry was assessed at age 8 years (6-11) in 4 different ways: body mass index (BMI z-score), fat mass (z-scores) determined from bioimpedence measurements, waist circumference (z-scores), and skinfold thickness (z-scores) determined as the sum of subscapular and triceps skinfold thickness. In addition, maternal pre-pregnancy BMI (kg/m²) was recorded. Relative telomere length was measured by using real time polymerase chain reaction (qPCR). Effect estimates were calculated using multiple linear mixed models with a random cohort effect adjusted for relevant covariates (i.e.; maternal education, child's age, sex, batch effect, birth weight, and child's ethnicity).

Results For each unit (1 kg/m²) increment in maternal pre-pregnancy BMI, the child's telomere length was 0.23% shorter (95% CI: 0.46, 0.01%). Each unit increase in child BMI z-score was associated with 1.21% (95% CI: 2.11, 0.30%) shorter telomere lengths. Inverse associations of borderline statistical significance were observed between child waist circumference and telomere length (-0.96% per z-score unit; 95% CI: -2.06, 0.16%), and skinfold thickness and telomere length (-0.10% per z-score unit; -0.23, 0.02%).

Conclusion This study demonstrates that children with higher obesity scores have shorter telomeres, and that child BMI was more strongly associated with shorter telomere length than maternal pre-pregnancy BMI. These findings suggest that obesity may accelerate telomere shortening in children and thus may accelerate cellular aging.

Introduction

The prevalence of obesity is rising rapidly throughout the world. Obesity is a common risk factor for increased morbidity and mortality in adulthood. Obesity has been consistently associated with increased systemic inflammation and oxidative stress [195, 196], which are also causes of telomere shortening in cells [68, 197]. Telomeres are nucleoprotein structures containing tandem repeats of DNA (5'-TTAGGG-3'), sited at the termini of the chromosomes [198]. Telomeres have significant function in maintaining the integrity of chromosomes and the stability of the genome, and to prevent end-to-end chromosomal fusions [199]. Telomeres shorten with each cell division because DNA polymerase is unable to fully replicate the 3' end of the DNA strand. Consequently, telomere length is considered a biomarker of biological aging and shorter telomeres have been associated with age-related diseases such as cardiovascular disease [60, 152, 153], type 2 diabetes [154] and increased mortality [155-157]. Telomere length variability and attrition rate has been explained by heritability and by different environmental determinants [138, 200-203]. In studies with adult subjects, obesity and other pro-inflammatory risk factors have been associated with shorter leukocyte telomere length [138, 203-206], suggesting that long-term exposure to these factors exacerbates telomere attrition. In children, however, case-control studies of telomere length and childhood obesity have produced conflicting results with obesity being related to shorter telomeres or not related to telomeres [207-209].

Further, maternal obesity is a well-known risk factor for adverse pregnancy outcomes [210], and is associated with childhood obesity [211], childhood asthma [212], and cardiovascular disease [213, 214]. Recent findings have shown that newborn telomere length may be influenced by several intrauterine effects, such as air pollution exposure [182, 215-219]. Additionally, Martens *et al* (2016) showed that pre-pregnancy BMI is associated with shorter newborn cord blood and placental telomeres [168].

The rate of telomere attrition is greatest in young children [159] and telomere length decline then continues at a slower rate throughout adulthood [160]. Consequently, telomere loss in childhood is a potentially important factor determining telomere length in adults, but little is known about the environmental exposures that impact on telomere attrition and molecular longevity during early life. The aim of the present study was to evaluate the effects of maternal pre-pregnancy BMI and child obesity parameters on telomere length measured in children aged 8 years. These analyses were carried out in a multi-centre European birth cohort study in six different European countries.

Methods

Study population and data collection

The Human Early-Life Exposome (HELIX) study represents a collaborative project across six established and ongoing longitudinal population-based birth cohort studies in Europe: the Born in Bradford (BiB) study in the UK [161], the Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) study in France [162], the INfancia y Medio Ambiente (INMA) cohort in Spain [163], the Kaunus cohort (KANC) in Lithuania [164], the Norwegian Mother and Child Cohort Study (MoBa) [165] and the RHEA Mother Child Cohort study in Crete, Greece [166]. The analysis of this paper made use of the HELIX subcohort. Eligibility criteria for inclusion in the subcohort were: a) age 6-11 years at the time of the visit, with a preference for ages 7-9 years if possible; b) sufficient stored pregnancy blood and urine samples available for analysis of prenatal exposure biomarkers; c) complete address history available from first to last follow-up point; d) no serious health problems that may affect the performance of the clinical testing (e.g spirometry) or impact the volunteer's safety (e.g. renal failure, pneumonia). Finally we had mother-child pairs with complete questionnaire and clinical examination data, and urine and blood samples.

Local ethical committees approved the studies that were conducted according to the guidelines laid down in the Declaration of Helsinki. All participating women provided informed written consent.

Each cohort collected detailed information on maternal age at birth, maternal education, maternal marital status, smoking status during pregnancy, parity, and maternal ethnicity from each study participant during pregnancy or at birth by questionnaire or medical records. The level of maternal education reported by the participant was used as the primary indicator of SES and categorized according to the International Standard Classification of Education (ISCED) [220] as three levels: "low" (Less than primary, primary, and lower secondary education, ISCED 2011 levels 0-2); "middle (Upper secondary and post-secondary non-tertiary education, ISCED 2011 level 3 and 4); high" (Tertiary education, ISCED 2011 levels 5-8). Maternal smoking status was categorized as "no active smoking during pregnancy" and "active smoking during pregnancy". Maternal ethnicity was defined for all cohorts and subdivided in 7 different groups (African, Asian, Caucasian, native-American, Pakistani, White-not European, or others). Perinatal parameters such as birth date, and newborn sex were obtained at birth.

Blood collection and DNA extraction

Buffy coat was collected in EDTA tubes. Leukocyte DNA was extracted using the Chemagen kit (Perkin Elmer) in batches of 12 samples. Samples were extracted by cohort and ultimately DNA concentration was determined in a NanoDrop 1000 UV-Vis Spectrophotometer (ThermoScientific) and with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies).

Average relative telomere length measurement

Average relative telomere length was measured by a modified qPCR protocol as described previously [167]. Telomere and single copy-gene reaction mixture

and PCR cycles used can be found in Martens et al [168]. 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.

Obesity parameters

Maternal anthropometrics included maternal pre-pregnancy BMI. Maternal height was measured and pre-pregnancy weight reported by the mother at the first trimester visit; these were used to calculate pre-pregnancy BMI (kg/m2). A BMI value within the range of 18.5-24.9 is categorized as normal, 25.0-29.9 as overweight and ≥ 30 as obese. Child's anthropometrics included child's BMI, waist circumference, skinfold thickness and fat mass. Briefly, height (cm) and weight (kg) were measured without shoes and with light clothing. World Health Organization reference curves were used to calculate age standardized z-scores for BMI, for each child adjusted for sex and exact age [221]. Waist circumference (cm) was measured with the child in a standing position using standardized procedures. Measurements were taken in direct contact with the skin at the top of the iliac crests, during minimal respiration. Waist circumference was standardized using internal z-score for age and sex. Skinfold thickness at four points were measured to the nearest 0.1 mm (triceps, subscapular, suprailiac, and quadriceps) following standardized procedures. The sum of skinfold thickness of 2 points (subscapular and triceps) has been found to be more sensitive and thus was used in this study. This sum was standardized using internal z-score for age and sex. Measurements of bioimpedance were taken by placing electrodes on cleaned

skin. Fat free mass was estimated based on a multiracial equation recently developed for children based on impedance values. From this equation body fat mass in kg was calculated. Ultimately, fat mass was standardized using internal z-score for age and sex.

Statistical analysis

Continuous data were checked for normality. Average relative telomere length showed a skewed distribution and were log10 transformed to improve normal distribution. The obesity parameters were treated as continuous variables for testing associations with telomere length. Generalized additive models (GAMs) were used to assess the linearity of the associations between the obesity parameters (maternal pre-pregnancy BMI, child's BMI, waist circumference, skinfold thickness and fat mass) and telomere length. Multiple linear models were applied to address the association between the obesity parameters (maternal pre-pregnancy BMI, child's BMI, waist circumference, skinfold thickness and fat mass) and telomere length. Covariates considered for entry in the model were maternal age at birth, maternal education, child's ethnicity, marital status, birth weight, child's sex, child's age in years, cohort, and batch. From these variable we selected those that changed the effect estimate of interest by greater than 10%. The adjusted model contained maternal education, child's age, sex, batch, cohort, child's ethnicity, and birth weight. We performed multiple imputation using chained equations to account for missing values of potential confounding variables, 20 datasets were generated and pooled for analyses (Supplemental Materials).

Since we use both maternal pre-pregnancy BMI as child BMI in our different models added an additional model in which we included both maternal pre-pregnancy BMI as child BMI. Additionally, we had stratified the associations between child BMI and leukocyte telomere length by maternal BMI group.

All the mixed models were performed using the SAS 9.3 statistical software (SAS Institute Inc. Cary, NC, USA).

Results

Characteristics of the study population

Table 1 describes the general characteristics of the study population (n = 1396). The children had a mean (SD) age of 8 (1.5) years, 753 (53.9%) were boy, they were mainly Caucasian (87.4%) and they had a mean (SD) BMI of 17.1 (2.7) kg/m². Mean (SD) maternal age at delivery was 30.5 (4.9) years and mean (SD) maternal pre-pregnancy BMI was 24.9 (5.1) kg/m². 643 (46.1%) mothers were highly educated, 635 (45.5%) of the mothers were primiparous and 229 (13.4%) of the mothers actively smoked during pregnancy. The characteristics for the individual cohorts are presented in the Supplemental Materials (Table S1). In summary, BIB had the lowest percentage caucasians (43%). The children in BIB, RHEA and KANC were the youngest (± 6.5 years), while the children in EDEN were the oldest (10.8 ± 5.8 years). The children in INMA had the highest BMI (18.1 ± 3.0 kg/m²), whereas children in BIB had the lowest BMI (16.0 ± 2.0 kg/m²). Mothers in MOBA were the oldest (32.8 ± 3.7 years) and were highly educated (77.0%) whereas mothers in BIB were the youngest (28.6 ± 5.8 years) and lower educated (42.9%). Additionally mothers in BIB had the highest pre-pregnancy BMI (28.3 ± 5.3 kg/m²), while mothers in MOBA had the lowest pre-pregnancy BMI (22.6 ± 3.1 kg/m²).

Table 2 shows the correlations between the different obesity parameters. Maternal prepregnancy BMI was significantly correlated with the different child obesity parameters. Child obesity parameters were highly associated with one another.

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Table 1. General characteristics of the complete case study population

	Mean (SD) or n (%)
Children	
Sex	
Girls	643 (46.1)
Boys	753 (53.9)
Ethnicity	
African	12 (0.9)
Asian	21 (1.5)
White European	1220 (87.4)
Native_American	13 (0.9)
Other	22 (1.6)
South-Asian	79 (5.7)
White_not European	26 (1.9)
Cohort	
INMA	428 (30.7)
MOBA	213 (15.3)
BIB	205 (14.7)
RHEA	202 (14.5)
KANC	199 (14.3)
EDEN	149 (10.7)
Gestational age	39.6 ± 1.6
Missings	12 (0.9)
Age, years	8.0 ± 1.5
BMI, kg/m²	17.1 ± 2.7
Relative telomere length	1.0 (0.9 – 1.1)
Mothers	
Age at delivery	30.5 ± 4.9
Missings	15 (1.1)
Pre-pregnancy BMI, kg/m²	24.9 ± 5.1
Education	
Low	219(15.8)
Middle	480 (34.4)
High	643 (46.1)
Missings	54 (3.4)
Parity	
1	635 (45.5)
2	498 (35.7)
≥3	228 (16.3)
Missings	35 (2.5)

Continuous covariates expressed by mean and standard deviation (SD) or geometric mean and 25–75th percentile; categorical covariates described by frequencies (%).

Table 2. Correlations between the different obesity parameters

	Maternal pre-pregnancy BMI	BMI z-score	Fatmass z-score	Waist circumference z-score	Skinfold z-score
Maternal pre-pregnancy BMI					
BMI z-score	0.27*				
Fatmass z-score	0.24*	0.81*			
Waist circumference z-score	0.22*	0.82*	0.81*		
Skinfold z-score	0.15*	0.77*	0.75*	0.71*	

[a]Spearman correlation coefficient between the different obesity parameters

*P-value < 0.0001

Association between obesity parameters and telomere length

Leukocyte telomere length was consistently lower in children with mothers who had a higher pre-pregnancy BMI (Table 3). For each unit (1 kg/m²) increment in maternal pre-pregnancy BMI, child's leukocyte telomere length was 0.23% shorter (95% CI: -0.46, -0.01%). Figure 1 shows the linear relationship of the association between child telomere length and maternal pre-pregnancy BMI and child BMI z-score.

Table 3. Obesity parameters and child's leukocyte telomere length

	n	% change	95% CI	P-value
Maternal pre-pregnancy BMI	1396	-0.23	-0.46,0	0.04
BMI z-score	1396	-1.21	-2.11,-0.3	0.01
Fatmass z-score	1365	-0.65	-1.76,0.48	0.26
Waist circumference z-score	1373	-0.96	-2.06,0.16	0.09
Skinfold z-score	1363	-0.10	-0.23,0.02	0.09

Estimates are presented as a percentage change in average relative telomere length for each kg/m2 BMI increase in maternal pre-pregnancy BMI or for each z-score increase in the child's anthropometric variable.

Models were adjusted for maternal education, child's age, sex, batch effect, child's ethnicity, and birth weight.

Inverse association were found between child's telomere length and four common anthropometric variables in the children, including BMI, waist circumference, skin fold thickness, and fat mass (Table 3). Each unit increase in BMI z-score was associated with 1.21% (95% CI: -2.11, -0.30%) shorter telomere length. Inverse borderline significant associations were observed between telomere length and waist circumference z-score (-0.96% per unit increase; 95% CI: -2.06, 0.16%), and skinfold thickness z-score (-0.10% per unit increase; -0.23, 0.02%). Finally, fat mass z-score was not significantly associated with telomere length (-0.65% per unit increase; 95% CI: -1.76, 0.48). Additionally, categorical analyses showed that compared to children with a normal weight, telomere length was lower in overweight and obese children (Table 4).

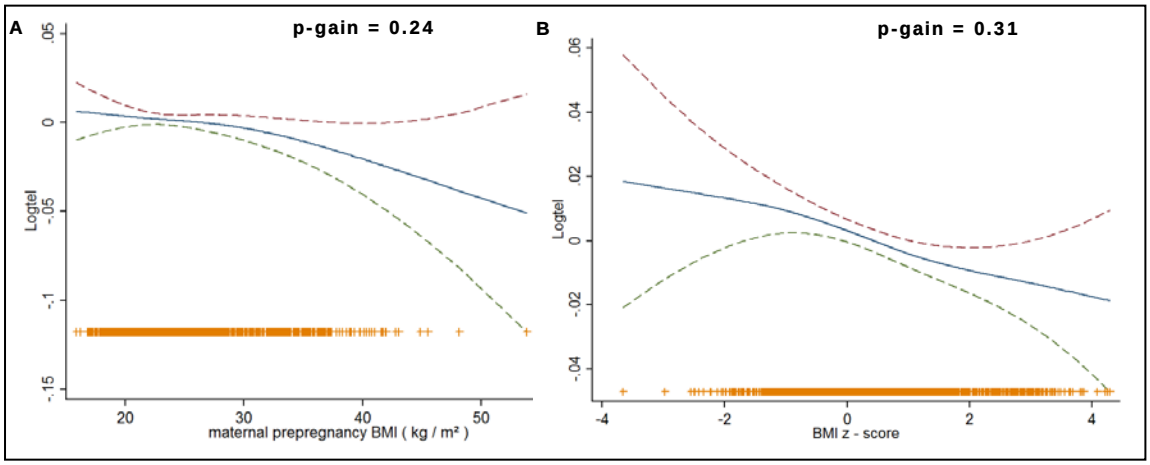

Figure 1. GAM models that show the relation between child telomere length and (A) maternal pre-pregnancy BMI and (B) child BMI

Table 4. Categorical association between child BMI and telomere length

Child BMI	n	% change	95% CI	P-value
Normal weight	1097	Ref		
Overweight	215	-2.53	-5.40, 0.43	0.09
Obese	84	-1.27	-5.65, 3.32	0.58
Overweight and obese	299	-2.18	-4.73, 0.36	0.09
Maternal pre-pregnancy BMI	**n**	**% change**	**95% CI**	**P-value**
Normal weight	853	Ref		
Overweight	336	-0.87	-3.56, 1.90	0.54
Obese	207	-2.20	-5.51, 1.23	0.21
Overweight and obese	543	-0.31	-2.62, 2.06	0.80

Estimates are presented as a percentage change in average relative telomere length for each kg/m2 BMI increase in maternal pre-pregnancy BMI or for each z-score increase in the child's BMI.
Models were adjusted for maternal education, child's age, sex, batch effect, child's ethnicity, and birth weight.

We have included an additional analysis in which we stratified the associations for child BMI by maternal BMI group (Table 5). The results show that children in the group of mothers with overweight and obesity had significant shorter telomere length (-1.36% per each BMI z-score unit increase; 95% CI: -2.58, -0.13). Moreover, adding maternal pre-pregnancy BMI and child BMI to the same model did not substantially change our reported associations (Table 6).

Table 5. Association between child BMI and telomere length stratified by maternal prepregnancy BMI

	n	% change	95% CI	P-value
Normal weight (BMI < 25 kg/m²)	854	-1.10	-2.41, 0.22	0.10
Overweight (BMI ≥ 25 kg/m² and BMI < 30 kg/m²	332	-0.88	-2.52, 0.79	0.30
Obese (BMI ≥ 30 kg/m²)	210	-1.13	-3.02, 0.79	0.25
Overweight and obese (BMI ≥ 25 kg/m²)	542	-1.36	-2.58, -0.13	0.03

Estimates are presented as a percentage change in average relative telomere length for each kg/m2 BMI increase in maternal pre-pregnancy BMI or for each z-score increase in the child's BMI.

Models were adjusted for maternal education, child's age, sex, batch effect, child's ethnicity, and birth weight.

Table 6. Association between BMI and telomere length in a model including both maternal prepregnancy BMI and child's BMI.

	n	% change	95% CI	P-value
Maternal prepregnancy BMI	1396	-0.12	-0.35,0.12	0.33
Child's BMI z-score	1396	-1.07	-2.01,-0.12	0.03

Estimates are presented as a percentage change in average relative telomere length for each kg/m2 BMI increase in maternal pre-pregnancy BMI or for each z-score increase in the child's BMI.

Models were adjusted for maternal education, child's age, sex, batch effect, child's ethnicity, and birth weight.

Discussion

Telomere length varies greatly between persons of the same age and this variation is present from early life. The key finding of this paper is that obesity parameters in children are associated with a shorting in their leukocyte telomere length, independent of maternal education, child's age, sex, batch effect, child's ethnicity, and birth weight. Additionally, we showed that maternal pre-pregnancy BMI is associated with shorter telomeres in children. The findings of this study deserve attention because they can be translated to premature aging as early as childhood.

The telomere loss in childhood may increase the risk for chronic diseases in adulthood. We were not able to estimate the effects of our decline based on absolute telomere length, since we used a real-time PCR method that cannot provide these absolute values. Nevertheless, an estimation can be based on available data from young adulthood telomere length, leading to an estimated loss of on average 8 kb (36). This indicates that a decrease of 1.21% leads to a loss of approximately 97 bp in LTL for each child BMI z-score unit increase. In adult leukocytes, the annual telomere loss was estimated between 32.2 and 45.5 bp (37), indicating that each child BMI z-score unit increase is equivalent

to a loss of 2.1 to 3.0 years (based on telomere attritions of 32.2-45.5 bp per year).

Previous studies investigating telomere length and childhood obesity were case-control studies. In a study of 148 Arab children mean telomere length was shorter in obese boys compared with lean boys [209], whereas, in a study of 53 Italian children no difference in telomere length was found between obese and non-obese individuals [207]. In another study conducted in 793 French children, obese children had a mean telomere length that was 24% shorter than that of non-obese children; However, when considering continuous BMI z-score, no association was observed. The authors proposed that this association is with absolute body size, rather than size relative to age-and gender-matched peers [208]. Our findings support the association between obesity parameters and telomere length in adults. Meta-analytical evidence suggests that leukocyte telomere length is inversely associated with BMI in adulthood [191]. In Chinese women, ages 40-70 years, BMI, waist circumference, and hip circumference were associated with shorter telomeres [222]. In a study encompassing 989 middle-aged individuals, a correlation was found between telomere length and obesity parameters [223]. In the Fels Longitudinal Study with 309 participants aged 8 to 80 years, BMI, waist circumference, hip circumference, total body fat, and visceral adipose tissue volume were all associated with shorter telomere length [224].Furthermore, a recent study of Martens et al. [168] showed that maternal pre-pregnancy BMI is associated with shorter newborn cord blood and placental telomeres. These findings shed light on the pre-pregnancy effects of maternal BMI on the next generation. In this paper we also looked at maternal pre-pregnancy BMI and child's telomere length to see if the pre-pregnancy association observed by Martens et al. may persist into childhood. We found that leukocyte telomere length was consistently lower in children with mothers who had a higher pre-pregnancy BMI, although this association was attenuated when we added child BMI to the model.

High waist circumference is normally considered as a risk factor for cardiovascular disease. The negative association found for BMI and waist

circumference and telomere length and the lack of association between fat mass and telomere length in this study is interesting. This implies a body shape effect independent of the fat percentage.

The mechanisms underlying the association between obesity and short telomeres are unknown. Obesity is regarded as a crucial factor in the regulation of adipose tissue aging and further metabolic outcomes such as insulin resistance, diabetes and cardiovascular disease [66, 225, 226]. A study of Minamino et al found that the p53 pathway in adipose tissue, which is the key in the aging process of adipose tissue and increased inflammation, may play an important role in the association between obesity and obesity-mediated aging [66]. This may be partially responsible for the observed inverse association with telomere length, as high levels of reactive oxygen species (ROS), produced by obesity, result in higher oxidative stress that is thought to accelerated shortening of telomeres in addition to cellular replication [68, 227]. Oxidative stress is a direct and indirect source of single strand breaks in DNA [68]. Compared to genomic DNA, telomeres contain G-rich fragments that are highly sensitive to ROS and make it an ideal target for oxidative damage, and telomeric DNA is relative less capable of DNA repair. Consequently, the higher levels of oxidative stress induced by obesity leads to breakage of DNA and a more rapid decline in telomere length [180].

We need to address some potential limitations of this study. Firstly, we used a real-time PCR method to determine telomere length, which has, in general a higher assay variability compared to the traditionally used TRF method [192, 193]. However, an inter-laboratory comparison of our method showed that the coefficient of variation was less than 7%. Secondly, the assessment of telomere length at 8 year of age represents only a snapshot in childhood. We were not able to evaluate telomere dynamics throughout the entire pregnancy and the childhood period. However, repeated measurement after 6 months in 150 children show a very high correlation ($r = 0.91$; $p < 0.001$) in the same kids. As overweight mothers may potentially have shorter telomeres, the

association between pre-pregnancy BMI and child telomere length might be mediated by maternal telomere length. This mediation could not be addressed as no data on maternal telomere lengths was available. Paternal age exerts a considerable effect on child telomere length [194], however, this data was not available in our cohorts. Finally, other potential important factors that occur during pregnancy and childhood, such as newborn telomerase activity and alteration of oxidative stress-related markers in mothers and children, which might influence child telomere length, were not measured. The major strengths of this study are that we used a large multi centre European study including 6 populations across Europe. Furthermore, we had a very thorough obesity assessment in children. We incorporated different obesity indicators in our study to take into account the different measures of body composition. BMI represents a good parameter to describe overweight and obesity, and waist circumference, skinfold thickness and fat mass add information at any level of BMI to get a better obesity prediction. This study is, to the best of our knowledge, the largest by far in assessing childhood obesity effects on telomere length.

In conclusion, we have demonstrated that children with higher BMI and higher scores of other obesity indicators have shorter telomeres and that child BMI was more strongly associated with shorter telomere length than maternal pre-pregnancy BMI. This is the largest multicentric study to report associations between obesity parameters in mothers and children and telomere length in children. Telomere length in early life predicts life span; therefore, further population-based studies in young cohorts are required to investigate if the difference in telomere length that we observe by maternal and childhood obesity status extends into adulthood. Prevention of maternal and child obesity may ultimately impact biological aging over the life span.

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Supplemental material

Supplemental Table S1. General characteristics of the complete case study population stratified by cohort

	INMA (n = 428)	MOBA (n = 213)	BIB (n = 205)	RHEA (n = 199)	KANC (n = 202)	EDEN (n = 149)
Children						
Sex						
Girls	206 (48.13)	98 (46.0)	93 (45.37)	89 (44.72)	92 (45.54)	65 (43.6)
Boys	222 (51.87)	115 (54.0)	112 (54.63)	110 (55.28)	110 (54.46)	84 (56.4)
Ethnicity						
African	5 (1.17)	0 (0.0)	7 (3.41)	0 (0.0)	0 (0.0)	0 (0.0)
Asian	2 (0.47)	6 (2.9)	13 (6.34)	0 (0.0)	0 (0.0)	0 (0.0)
White European	380 (88.32)	204 (95.7)	89 (43.41)	199 (100.0)	202 (100.0)	149 (0.0)
Mixed native American	11 (2.57)	2 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other	4 (0.93)	1 (0.4)	17 (8.29)	0 (0.0)	0 (0.0)	0 (0.0)
South-Asian	0 (0.0)	0 (0.0)	79 (38.54)	0 (0.0)	0 (0.0)	0 (0.0)
White not European	26 (6.07)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Gestational age, weeks	39.9 ± 1.4	40.1 ± 1.7	39.7 ± 1.8	38.4 ± 1.4	39.4 ± 1.3	39.8 ± 1.7
Child age, years	9.02 ± 0.65	8.5 ± 0.5	6.6 ± 0.2	6.5 ± 0.3	6.5 ± 0.5	10.8 ± 0.6
Mothers						
Age at delivery	31.5 ± 4.2	32.8 ± 3.7	28.6 ± 5.8	30.9 ± 4.8	28.57 ± 5.0	30.7 ± 5.0
Missings	1 (0.2)	6 (2.8)	1 (0.5)	2 (1.0)	2 (1.0)	0 (0.0)
Education						
Low	99 (23.1)	0 (0.0)	88 (42.9)	9 (4.5)	12 (5.9)	11 (7.4)
Middle	174 (40.7)	41 (19.2)	31 (15.1)	111 (55.8)	69 (34.2)	55 (36.9)
High	141 (32.9)	164 (77.0)	64 (31.2)	79 (39.7)	116 (57.4)	83 (55.7)
Missings	14 (3.3)	8 (3.8)	22 (10.7)	2 (1.0)	5 (2.5)	3 (2.0)
Active smoking during pregnancy						
Yes	109 (225.46)	9 (4.4)	25 (12.2)	43 (21.6)	13 (6.44)	31 (20.8)
No	311 (72.66)	198 (91.6)	157 (76.6)	156 (78.4)	184 (91.09)	118 (79.2)
Missings	8 (1.87)	9 (4.4)	23 (11.2)	1 (0.5)	5 (2.5)	0 (0.0)
Parity						
1	230 (53.7)	93 (43.7))	83 (40.5)	74 (37.2)	84 (41.6)	71 (47.7)
2	165 (38.6)	86 (40.4)	52 (25.4)	85 (42.7)	59 (29.2)	51 (34.2)
≥3	28 (6.5)	28 (13.1)	56 (27.3)	35 (17.6)	54 (26.7)	27 (18.1)
Missings	5 (1.2)	6 (2.8)	14 (6.8)	5 (2.5)	5 (2.5)	0 (0.0)

Continuous covariates expressed by mean and standard deviation ± SD; categorical covariates described by number and frequencies (%).

Supplemental Table S2. Exposure characteristics of the complete case study population stratified by cohort

				Percentile				
	n	**Mean**	**SD**	**5th**	**25th**	**50th**	**75th**	**95th**
INMA								
NO_2 Prenatal	351	43.23	11.17	24.55	36.08	43.24	49.07	60.83
NO_2 Postnatal	401	33.04	11.81	11.74	26.83	35.4	40.69	50.18
$PM_{2.5}$ Prenatal	351	15.08	1.72	12.3	14.1	14.97	15.98	17.86
$PM_{2.5}$ Postnatal	401	13.31	1.72	10.47	12.66	13.3	13.88	15.7
MOBA								
NO_2 Prenatal	206	20.51	7.67	11.17	14.49	18.52	25.24	36.31
NO_2 Postnatal	207	26.2	5.41	19.35	22.72	25.44	29.59	33.6
$PM_{2.5}$ Prenatal	207	12.06	2.22	8.13	10.47	12.12	13.53	15.94
$PM_{2.5}$ Postnatal	207	8.12	1.61	5.95	7.06	7.77	9.06	11.13
BIB								
NO_2 Prenatal	205	20.79	3.43	15.66	18.43	20.61	23.12	26.71
NO_2 Postnatal	205	31.6	3.93	26.68	28.61	31.29	33.67	38.11
$PM_{2.5}$ Prenatal	205	14.37	1.78	11.49	13.28	14.18	15.48	17.5
$PM_{2.5}$ Postnatal	205	14.39	1.2	12.66	13.58	14.23	15.12	16.44
RHEA								
NO_2 Prenatal	199	12.14	4.21	8.34	9.28	11.19	12.8	21.83
NO_2 Postnatal	199	10.99	3.47	7.66	6.87	10.09	12.05	18.72
$PM_{2.5}$ Prenatal	199	14.49	1.24	12.95	12.95	14.39	15.26	16.99
$PM_{2.5}$ Postnatal	199	14.09	1.86	11.71	12.83	13.63	15.12	17.47
KANC								
NO_2 Prenatal	195	18.53	3.74	13.42	15.94	17.83	20.67	24.79
NO_2 Postnatal	194	13.99	2.51	10.05	12.51	13.99	15.21	17.8
$PM_{2.5}$ Prenatal	195	17.61	2.44	13.49	15.78	17.98	19.09	20.93
$PM_{2.5}$ Postnatal	194	18.29	1.6	15.28	17.58	18.28	19.34	20.68
KANC								
NO_2 Prenatal	80	15.06	5.16	9.94	11.65	13.38	17.32	24.28
NO_2 Postnatal	148	8.3	1.96	6.44	6.74	7.71	9.13	12.56
$PM_{2.5}$ Prenatal	149	18.09	1.54	15.52	17.12	18.09	18.99	20.87
$PM_{2.5}$ Postnatal	148	10.68	0.44	10.17	10.39	10.62	10.87	11.38

Continuous variables expressed by mean and standard deviation (SD)

Chapter 6.

General discussion

Aging is a complex physiological phenotype, responsive to a plethora of environmental stressors from early life onwards. According to the Developmental Origins of Health and Disease (DOHaD) small changes in the early life environment shape the future probability of the development of age-related diseases.[1, 88]

In this doctoral dissertation, we assessed the effect of early life exposure to pro- inflammatory risk factors on mitochondrial DNA (mtDNA) content and telomere length, considered as markers of biological aging, at birth and during childhood. Furthermore we investigated if placental mtDNA content was an intermediate or modulating factor between air pollution exposure and infant growth. For these purposes, we used 3 different birth cohort studies: The Belgian ENVIRONAGE (ENVIRonmental influence ON AGEing in early life) birth cohort study, the Spanish INMA (INfancia y Medio Ambiente; Environment and Childhood) birth cohort study and the multi-centre European birth cohort study HELIX (Human Early-Life Exposome). The main findings of this doctoral dissertation are presented in Table 1.

The novelties of this dissertation include:

- The evaluation of placental mtDNA content as an intermediate factor of the association between prenatal NO_2 exposure and birth weight and infant growth
- The assessment of the associations between NO_2 and PM exposure and telomere length in childhood
- The investigation of the association between obesity parameters and telomere length in childhood

Table 1. Main findings of the doctoral dissertation

Chapter	What is known	What this study adds	Perspectives and conclusions
Chapter 2	• Placental mitochondria play an important role in the proper formation and function of the placenta • Mitochondrial DNA (mtDNA) content is a molecular marker of mitochondrial damage, oxidative stress and inflammation • Air pollutants can induce oxidative stress and inflammation	• Prenatal NO_2 exposure is associated with: - Lower birth weight - Lower placental mtDNA content • Placental mtDNA content was associated with higher mean birth weight • 10% of the association between prenatal NO_2 exposure and birth weight was mediated by changes in placental mtDNA content	• Our findings will contribute to the understanding of molecular pathways underlying the association between prenatal air pollution exposure and low birth weight • Alterations in placental mtDNA content can mediate the association between NO_2 exposure and birth weight
Chapter 3	• The association between prenatal air pollution exposure and postnatal growth has hardly been explored • Infant growth is believed to be a continuation of *in utero* growth and is influenced predominantly by factors determining intra-uterine growth and nutrition • Changes in placental mtDNA content may represent a biological effect along the path linking air pollution to effects on the infant	• Prenatal NO_2 exposure in early pregnancy was associated with height at 6 months of age and weight at 1 year of age • The associations between prenatal NO_2 exposure and height and weight at 6 months and 1 year of age were mediated by birth length and birth weight • 5.5% of the association between early pregnancy NO_2 exposure and length at 6 months of age could be mediated by placental mtDNA content	• This study suggests that impaired fetal growth caused by prenatal air pollution exposure can lead to impaired infant growth during the first year of life • Molecular adaptations in placental mtDNA are associated with postnatal consequences of air pollution induced alterations in growth

Table 1. Main findings of the doctoral dissertation (Continued)

Chapter	What is known	What this study adds	Perspectives and conclusions
Chapter 4	• Telomere length varies greatly between persons of the same age and this variation is present from early life • Telomeres shorten which each cell division and is considered a marker of biological aging • The natural erosion of telomeres can be accelerated through oxidative stress and inflammation induced by air pollution exposure	• Prenatal and postnatal NO_2 exposure was associated with shorter leukocyte telomere length in 8 year old children • Residential proximity to nearest major road during childhood was associated with shorter telomere length	• Our findings suggest that healthy air during early life is associated with a favorable biomolecular longevity in children • Our evidence of biomolecular harm helps elucidate causal pathways between air pollution and later adverse health outcomes • Adequate reduction of traffic related air pollution levels may promote molecular longevity from early life onwards
Chapter 5	• Shorter telomeres and obesity have both been associated with age-related diseases such as cardiovascular disease and type 2 diabetes • In recent years, obesity has been associated with shorter telomeres in adults	• Child obesity parameters were associated with a shortening in leukocyte telomere length in 8 year old children • Maternal pre-pregnancy BMI was associated with shorter telomeres in 8 year old children	• This study demonstrates that children with a higher obesity score and/or with mother with a higher pre-pregnancy BMI have a significant greater biological age than those with a lower obesity score and/or with mothers with a lower pre-pregnancy BMI • Our results highlight the importance of intervention that may impact the future life by decreasing comorbidities in adulthood

1. Discussion of the study findings

1.1 Air pollution and infant growth outcomes

The fetus and the infant are especially vulnerable to ambient air pollutants due to their differences in exposure, physiological immaturity and long life expectancy after exposure, compared to adults.[122] There are two different ways in which maternal air pollution exposure during pregnancy may affect the fetus: 1) directly, after translocation of the air pollutants via the mother's bloodstream to the placenta or into the amniotic fluid, and 2) indirectly, through mediation by inflammatory effects on the mother's cardiorespiratory system.[95, 228] Numerous studies have shown that maternal ambient air pollution exposure is associated with low birth weight, intra-uterine growth retardation, and preterm birth, even at low levels of air pollution.[29, 30, 229, 230] We assessed the association between prenatal NO_2 exposure and birth weight and length among 926 children from both the INMA and ENVIR*ON*AGE birth cohorts. Birth weight showed a decrease by 47.5 g for respectively a 10 µg/m³ increment in NO_2 exposure during the entire pregnancy. Furthermore, our study documented a significant decrease of 0.29 cm in length of the newborn for each 10 µg/m³ increment in prenatal NO_2 exposure.

Little is known about how these intra-uterine effects may translate into variations in growth patterns of children after birth. Using data from 336 INMA children we found that air pollution exposure during the beginning of pregnancy is significantly associated with a decrease of -6.6% in height at six months of age and -4.2% in weight at 1 year of age. These associations were mediated by birth length (31.7%; 95%CI: 34.5, 14.3) and weight (53.7%; 95%CI: 65.3, −0.3), respectively. This relative novel aspect of our study indicates that infant growth can be influenced by factors determining intra-uterine growth and nutrition.

In contrast to the epidemiological evidence, the mechanisms responsible for fetal growth restriction due to air pollution are largely unknown. Hypotheses are that air pollutants could cause oxidative stress, inflammation, blood

coagulation, endothelial function, and hemodynamic responses which all have an important effect on placental function.[131] The mechanism by which air pollutants can elicit placental inflammation and oxidative stress remain unclear. The maternal and fetal circulation are separated by the placental barrier that is formed by the syncytiotrophoblast layer, which faces the maternal environment.[228] This barrier contains placental transporter that can block or facilitate foreign compounds.[94, 228] It is believed that air pollutants can translocate into the mother's blood circulation after inhalation into the lungs, be transported to the placenta and influence placental growth and function.[228] The placenta plays a unique role in the transfer of gases, nutrients, and waste between the mother and developing child, and is therefore a key determinant of fetal growth.[93]

1.2 Age-related biomarkers

Aging is a complex physiological phenomenon. Aging begins at the very beginning of life, to accelerate at middle-age. The biological underpinnings of aging may begin in early life. Indeed, complications in adults often find their origin in risk factors operating in early life.[1] In this thesis, I focused on the biological markers of aging: i.e. mitochondrial DNA (mtDNA) content and telomere length.

1.2.1 Mitochondrial DNA content

MtDNA is vulnerable to oxidative damage due to the lack of protective histones and less efficient DNA repair system compared to nuclear DNA.[231] With advancing age, mutations and oxidative damage, mitochondrial DNA accumulates and causes a decrease in functionality.[232] We and others provide evidence linking mtDNA content to different environmental exposures in different population segments. In this dissertation, we showed an inverse association between NO_2 exposure and placental mtDNA content in early life.

Evidence on mtDNA content in relation to environmental exposure is still limited with inconsistent results. Exposure to PM air pollutants was associated with an increase [74], a decrease [137, 138], and no change in mtDNA content [135] in adults and elderly. Short-to-moderate-term ambient black carbon levels [139] and benzene exposure [140] in adults has been associated with an increase in mtDNA content. Studies investigating the effect of ambient air pollution exposure during pregnancy on placental mtDNA content are limited to maternal tobacco smoke exposure [134, 141]. Although, recently a significant inverse association between prenatal $PM_{2.5}$ exposure and lower mtDNA content in cord blood was found [142].

As shown above, environmental exposures have been reported to result in both decreased and increased mtDNA content. This may not only depend on the kind of environmental factor, but also on the dose and time point (short-term or chronic), the tissue assessed, oxidative stress level, and cell antioxidant capacity.[143, 144] Increased oxidative stress has a dual influence on mtDNA content. The current hypothesis is that mild oxidative stress may stimulate synthesis of mtDNA copy number and abundance as a compensatory mechanism. As a result, oxidative stress levels will increase and may result in decreased or no synthesis of mitochondria due to severe oxidative damage in cells [71]. Taken this hypothesis into account, a study in smokers found that the relative mtDNA content was increased in the lung tissues of light smokers but significantly decreased in heavy smokers [145].

1.2.1.1 mtDNA content as a mediator between exposure and outcome

mtDNA homeostasis is influenced by both genetic and environmental factors. Lifestyle factors and genetic host factors may play an important role in predicting susceptibility to air pollution.[81] We showed that air pollution is associated with mtDNA content. Therefore, we aimed to determine whether mtDNA content is a mediator of air pollution induced effects on infant growth.

In this dissertation we showed the importance of mtDNA content as a mediator between prenatal exposure to NO_2 and infant growth (Table 1). We showed that *in utero* NO_2 exposure was associated with a decreased placental mtDNA content. Further, NO_2 exposure during pregnancy was associated with a significant decrease in birth weight, birth length and length at 6 months of age. Additionally, placental mtDNA content was significantly and positively associated with birth weight, birth length and length at 6 months of age. Ultimately, we found significant mediated effects of mtDNA content in the associations between prenatal NO_2 and birth weight, birth length and length at 6 months of age.

1.2.2 Telomere length

Telomeres are ribonucleoprotein complexes that cap the end of chromosomes and thereby provide stability and protection to the coding DNA.[50] Telomeres shorten after each cellular division due to the end-replication problem.[51] This natural erosion of telomeres by chronological aging can be accelerated or delayed by several genetic and environmental factors, and the interaction between them. Telomeres are highly sensitive to oxidative stress due to their high guanine content and the deficient repair system of single-strand breaks.[67] Environmental factors appear to overrate the contribution of the end-replication problem partly through oxidative stress.

In this dissertation we found an inverse significant association between air pollution exposure and telomere length in 8 year old children. We found a -1.5% (95% CI -2.8, -0.2) decrement in childhood leukocyte telomeres for each SD increase in prenatal NO_2. The corresponding telomere shortening estimates for postnatal NO_2 exposure was -1.6% (95% CI: -2.9, -0.4) and for $PM_{2.5}$ -1.4% (95% CI: -2.9, 0.1).

In adults, airborne benzene and toluene, as indicator of traffic exposure, were associated with a decrease in telomere length.[184] An increase in airborne benzene and toluene exposure level equal to the difference between the 25th

and 75th centile was associated with -6.4% (95% CI: -10.4, -2.1) and -6.2% (95% CI: -10.4, -1.7) shorter leukocyte telomere length, respectively [184]. Among 165 never smoking adults, an IQR increase in annual black carbon was associated with -7.6% (95% CI: -12.8, -2.1) shorter telomeres.[183] A study among non-smoking elderly showed that long-term exposure to PM2.5 was associated with -16.8% (95% CI: -26.0, -7.4) shorter telomeres.[138]

However, in children the evidence of telomere length in relation to environmental exposure is limited. There is one study of school children in London in which they showed that annual air pollution exposure was associated with longer telomeres in saliva.[186] Our findings in children are in line with one previous study in newborns. In 641 newborns of the ENVIR*ON*AGE birth cohort, cord blood and placental telomere length were inversely significantly associated with $PM_{2.5}$ exposure during mid-gestation.[182]

In contrast to telomere shortening by long-term air pollution exposure, short-term air pollution exposure can lead to rapid increases in telomere length. In adults, a study among non-smoking elderly showed that short-term exposure to $PM_{2.5}$ was associated with increased telomere length.[138] Acute exposure to metal-rich PM exposure was positively associated with leukocyte telomere length in steel workers [233]. An increase of telomerase activity in lymphocytes and a clonal expansion of subpopulations of lymphocytes with longer telomeres following acute exposures have been suggested as potential underlying mechanisms [137, 138, 233].

1.2.2.1 Obesity parameters and telomere length

Obesity increases the risk for several non-communicable diseases such as diabetes mellitus, cardiovascular and fatty liver disease, and cancer. Increasing obesity rates poses a major public health challenge and will have considerable financial implications for the health system. Obesity is characterized by the presence of excessive adipose tissue which is identified

to increase systemic inflammation and oxidative stress, which interact with telomere attrition.

In this dissertation we investigated the effect of childhood obesity on telomere length in 8 year old children. We have demonstrated that children with higher obesity scores had shorter telomeres. For each unit (1 kg/m²) increment in maternal pre-pregnancy BMI, child leukocyte telomere length was 0.23% shorter (95% CI: -0.46, -0.01%). Each unit increase in child BMI z-score was associated with -1.21% (95% CI: -2.11, -0.30%) significant shorter telomere length. Our findings in children support the association between obesity and telomere length in adulthood. In the Fels Longitudinal Study with 309 participants aged 8 to 80 years, BMI, waist circumference, hip circumference, total body fat, and visceral adipose tissue volume were all inversely associated with telomere length [224]. In adult women, Valdes et al [204] reported shorter telomeres in obese women BMI > 30 compared with lean women which corresponds to an age difference of 8.8 years. In a study encompassing 989 middle-aged individuals, a significant or borderline non-significant correlation was found between telomere length and obesity parameters [223].

2. Implication of the presented work for public health

We observed that an adverse early environment not only resulted in adverse infant growth but also in lower mtDNA content and in shorter telomere length. In the literature, all changes in these aging markers are linked to disease outcome.

Birth weight has been associated with several health problems throughout life. The Developmental Origins of Health and Disease (DOHaD) hypothesis, often called the 'Barker hypothesis', states that suboptimal intrauterine conditions may alter fetal programming during critical periods of growth resulting in increased disease risk in adulthood.[109] Low birth weight has been associated with an increased risk of insulin-resistance syndrome [234], neurobehavioral

problems [235], hypertension [236], cardiovascular [89, 237], metabolic [238], and renal disease [89] in later life. Therefore, the public health impact of reductions in the studied infant outcomes is not limited to childhood, but projects into adulthood.

The consequences of an altered placental mtDNA content in later life are currently unknown. However, a decrease in mtDNA content has been related to the development of multiple forms of aging-related disease as type 2 diabetes [77, 135], and breast cancer [136].

Telomere length is considered as a marker of the biological aging process [51] and a general risk factor for several aging-related diseases in adults and elderly. Longitudinal studies in adults showed that telomere ranking is stable during adulthood [239]. This may suggest that the effects in adulthood also remains during adults life. Shorter telomeres have been associated with aging-related diseases such as cardiovascular disease [60, 152, 153], type 2 diabetes [154], cancer [240, 241], and increased mortality [155-157]. Furthermore, it is believed that the natural erosion of telomeres is accelerated through oxidative stress and inflammation [158].

3. Strengths and limitations

In this PhD dissertation we made use of epidemiological birth cohort studies. Birth cohort studies with follow-up across the life span have the enormous potential to help in the understanding of the etiology of numerous health conditions. An advantage of birth cohort research is the longitudinal follow-up of the cohort with follow-up that could continue indefinitely. The importance of continued follow-up well into adulthood is noted, because life course studies are needed to recognize that early life events can influence adult health and development. Since fetal life is thought to be among the most important critical periods of development, pregnancy has been the logical starting place for collecting data throughout the life course.

Epidemiological cohort studies have many more strengths. These studies follow a group of healthy people with different exposure levels and assess what happens to their health over time. The advantage of these studies is that the exposure become before the disease occurs which is necessary to establish possible causation. However, it is important to recognize that causality cannot be established definitely through epidemiological studies; nonetheless, these studies are powerful tools that can provide important evidence to suggest causality and to give information regarding the strength of an association between an exposure and an outcome in real life circumstances. Moreover, they make translation towards public health significance possible.

Another advantage in birth cohort studies is that the exposure precedes the outcome. This allows us to clearly determine the temporal relationship between exposure and outcome. Additionally, it also avoids certain types of selection bias. Thus, knowledge of the outcome status cannot influence the way subjects are selected.

We also have to take into account some potential limitations. A limitation of epidemiological studies is the possibility of bias due to confounding. A confounder is an unobserved exposure associated with the exposure of interest and is a potential cause of the outcome of interest. In other words, confounding occurs when exposure would remain associated with outcome even if all exposure effects were removed.[242, 243] Possible sources of confounding in this doctoral dissertation include participants demographic, socioeconomic, genetic, lifestyle characteristics of the participants, and methodological aspects (e.g. time of blood sampling and batch effect). To limit the risk of confounding in our analyses, we took several precautions. Based on the literature we identified a set of potential confounders that were included in our models. However, despite taken these confounders into account, we cannot exclude confounding due to variables that were inadequately measured, not considered, or imprecisely corrected for.

Another potential source of bias is error in the measurements of exposure. It may have many possible causes, including recall bias in self-administered questionnaires, imprecision of laboratory techniques, incomplete information in medical records, wrongly conducted physical measurements or the use of a measurement of a single point in time or space when the total exposure is of interest. In this PhD dissertation there is a potential of error in exposure measurements because the air pollution results are based on exposure at the home address, and potential exposure misclassification may be present because we could not account for other exposure sources that contribute to personal exposure, such as exposure during commute, at work or school, and elsewhere.

Error in the outcome measurement represents another problem in epidemiological research. The instruments to measure outcome should be both valid and reliable. In this PhD dissertation we used validated laboratory techniques and for the infant growth outcomes, quality assessments were performed to lower the risk of information bias.

An additional drawback of observational studies is that participants are not exposed to well-specified air pollutants during specific time windows of interest. In this doctoral dissertation, we assessed different exposure time windows, but the high correlation between these windows makes it difficult to identify the most vulnerable exposure period.

4. Future perspectives and valorization

In this dissertation we hypothesized that early-life exposure to air pollution and obesity may result in aging-related disease development later in life. This emphasizes the importance of the early-life environment. The study populations used were too young at follow-up to assess the development of aging-related diseases. Consequently, population-based studies with additional

follow-ups at a more advanced age could provide evidence to support our hypothesis.

We observed that an adverse early environment not only resulted in adverse infant growth but also in lower mtDNA content and shorter telomeres. As described earlier, all these changes are linked in the literature to disease outcome. However, the underlying biological pathway are not fully understood. Future research should assess if these outcomes all share one common pathway or if they are the result of different mechanisms. Additionally, further research should assess if the association between early environmental exposures and aging-related diseases is mediated by telomere length.

The reported results of this dissertation can help policy makers in taking measures to build a healthier living environment, protect human health and improve the air quality. These measures are very important since ambient air pollution is, according to the European Commission, responsible for 406,000 annual premature deaths in 2010 in the European Union, making it the number one environmental cause of death in this region [244]. Moreover, the Organization of Economic Cooperation and Development (OECD) estimated the cost of deaths in 24 European OECD countries (21 EU member-states plus Switzerland, Iceland and Norway) at 661,308 million euros [245]. WHO analyzed the effect of combustion-related particulate matter on life expectancy which indicated that current exposure to particulate matter from anthropogenic sources leads to an average loss of 8.6 months of life expectancy in Europe [4].

Environmentally friendly behavior is promoted by using several legal instruments. As an attempt to minimize air pollution, the European Commission enacted the 2005 Cleaner Air Directive [246]. This directive sets the EU limit values for NO_2 on 40 µg/m³ as an annual mean limit value and 200 µg/m³ as a hourly limit value [246].

Our research highlights the importance of reducing air pollution in the early-life environment. The NO_2 exposure in our studies were lower than the EU limit value (40 µg/m³). Our data shows that this limit is still too high, since we find

significant effects of NO_2 exposure on infant growth and age related biomarkers. This suggests that additional international cooperation in Europe is required to further reduce air pollution and to lower the NO_2 limit value. This reduction in air pollution will not only reduce mortality and disease development, but may also improve healthy aging.

5. Conclusions

In this PhD dissertation, we investigated the association between environmental exposure and age related biomarkers in newborns and children. Additionally, we assessed the association between air pollution exposure and growth in newborns and infants. In newborns we concentrated on NO_2 exposure, whereas in children we looked at both air pollution and obesity as environmental exposures.

We found evidence of an inverse association between prenatal NO_2 exposure and both placental mtDNA content and birth weight. Furthermore, we found evidence that suggests that prenatal air pollution exposure can lead to impaired infant growth that is determined by intra-uterine growth. Additionally, NO_2 induced alterations in placental mtDNA content might have consequences to growth up to six months of age.

Our analysis in a large European cohort study showed that NO_2 exposure in early life was inversely associated with telomere length. Additionally, a doubling of the residential proximity to nearest road during childhood was associated with shorter telomeres.

We have demonstrated that children with higher obesity scores have shorter telomeres.

The presented results emphasize the importance of children as a susceptible subgroup for the adverse effects of air pollution. Air pollution-induced health effects are not only limited to persons with underlying diseases or elderly, but also affect the individual from conception onwards. Telomere length in early life

predicts lifespan; therefore, further population-based studies in young cohorts are required to investigate the extent to which the reported differences in telomere length caused by early life exposure to environmental stressors extend into adulthood. Further research is also necessary to determine the clinical consequences of changes in mtDNA content and telomere length in early life. Since children with a higher environmental exposure have a greater biological age than those with a lower environmental exposure, the importance of intervention that may impact the future life by decreasing comorbidities in adulthood is highlighted.

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Curriculum Vitae

Diana Clemente Batalha Pardal was born in Genk (Belgium) on March 20th 1990. In 2008, she graduated from secondary school at Sint-Jan Berchmanscollege in Genk and started her study in Biomedical Sciences at Hasselt University. She followed the Master Biomedical Sciences – Environmental Health Sciences and she went to the Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain for 5 months to work on her masterthesis. She graduated in 2013 at Hasselt University. In the same year, she started her joint PhD in molecular epidemiology at the Centre for Environmental Sciences at Hasselt University (Prof. dr. Tim Nawrot) and the Institute for Global Health (ISGlobal) at the University Pompeu Fabra, Barcelona, Spain. As part of this joint PhD, she went 2 years to ISGlobal in Barcelona to work for and with the multi-centre European birth cohort study HELIX. She presented her results at several conferences including ISEE in Barcelona and Rome, Health Living in Maastricht, and DOHaD in Rotterdam.

List of publications

International peer-reviewed publications

1. Martens DS, Cox B, Janssen BG, **Clemente DBP**, Gasparrini A, Vanpoucke C, *et al.* 2017. Prenatal air pollution and newborns' predisposition to accelerated biological aging. JAMA pediatrics.
2. Janssen BG, Madlhoum N, Gyselaers W, Bijnens E, **Clemente DBP**, Cox B, *et al.* 2017. Cohort Profile: The ENVIRonmental influence ON early AGEing (ENVIRONAGE): a birth cohort study. Int J Epidemiol.
3. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iniguez C, *et al.* 2017. Prenatal ambient air pollution exposure, infant growth and placental mitochondrial DNA content in the inma birth cohort. Environ Res 157:96-102.
4. **Clemente DBP**, Casas M, Vilahur N, Vrijheid M, Sunyer J, Nawrot TS *et al* 2016. Prenatal Ambient Air Pollution, Placental Mitochondrial DNA Content, and Birth Weight in the INMA (Spain) and ENVIRONAGE (Belgium) Birth Cohorts. Environ Health Perspect 124(5): p. 659-665.

Abstracts

1. **Clemente DBP.** The exposome and telomere length. HELIX Scientific symposium: New Horizons for Early Life Exposome Research, Barcelona, Spain 2017 (Oral presentation)
2. **Clemente DBP**, Chatzi L, Danileviciute A, Fossati S, Maitre L, McEachan RRC, Meltzer H, Petraviciene I, Slama R, Thomsen C, Vafeiadi M, Wright J, Nawrot TS, Vrijheid M. Increased obesity parameters are associated with shorter telomeres in 8 year old children. Developmental Origins of Health and Disease (DOHaD), Rotterdam, the Netherlands 2017 (Oral presentation)
3. **Clemente DBP**, Vrijheid M, Martens DS, Chatzi L, Cirach M, Danileviciute A, Grazuleviciene R, Maitre L, McEachan RRC, Meltzer H, Tamayo I, Thomsen C, Vafeiadi M, Wright J, Slama R, Nieuwenhuijsen M, Nawrot TS. Exposure to ambient air pollution predicts telomere length in 8-year old children. Developmental Origins of Health and Disease (DOHaD), Rotterdam, the Netherlands 2017 (Poster presentation)
4. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Prenatal air pollution exposure, infant growth and placental mtDNA content in the INMA birth cohort. ISEE, Rome, Italy 2016 (Oral presentation)
5. **Clemente DBP**, Vrijheid M, Martens DS, Chatzi L, Cirach M, Danileviciute A, Grazuleviciene R, Maitre L, McEachan RRC, Meltzer H, Tamayo I, Thomsen C, Vafeiadi M, Wright J, Slama R, Nieuwenhuijsen M, Nawrot TS. Prenatal exposure to ambient air pollution predicts telomere length and mitochondrial DNA content in 8-year old children. ISEE, Rome, Italy 2016 (Oral presentation)
6. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Prenatal air pollution exposure, infant growth and placental mtDNA content in the INMA birth cohort. 13ª Jornadas Científicas INMA, Barcelona, Spain 2016 (Oral presentation)
7. **Clemente DBP**, Vrijheid M, Martens DS, Chatzi L, Cirach M, Danileviciute A, Grazuleviciene R, Maitre L, McEachan RRC, Meltzer H, Tamayo I, Thomsen C, Vafeiadi M, Wright J, Slama R, Nieuwenhuijsen M, Nawrot TS. 13ª Jornadas Científicas INMA, Barcelona, Spain 2016 (Oral presentation)
8. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Prenatal air pollution exposure and growth: The role of placental mtDNA content. 2nd ISEE Europe's Young Researchers Conference on Environmental Epidemiology, Utrecht, the Netherlands 2015 (Oral presentation)
9. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Prenatal air pollution exposure and growth: The role of

placental mtDNA content. Epidemiology Symposium, Leuven, Belgium 2015 (Poster presentation)

10. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Birth Weight and Ambient Air Pollution: the Role of Mitochondrial DNA Content. European Congress of Epidemiology – Healthy Living, Maastricht, The Netherlands 2015 (Poster presentation)

11. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Prenatal air pollution exposure and growth: The role of placental mtDNA content. INMA Scientific Conference, Barcelona, Spain 2015 (Oral presentation)

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14. **Clemente DBP**, Casas M, Janssen BG, Lertxundi A, Santa-Marina L, Iñiguez C, Llop S, Nawrot TS, Vrijheid M. Placental mitochondrial DNA-content in association with outdoor air pollution during *in utero* life and its potential role as mediator between birth weight and air pollution. Jornadas Científicas INMA, San Sebastián, Basque Country, Spain 2013 (Oral presentation by Casas M)