PhD thesis presented on 7 May 2018 at Hasselt University

Members of the Jury

Prof. Dr M. Ameloot, Hasselt University, Diepenbeek, Belgium, Chair

Prof. Dr T.S. Nawrot, Hasselt University, Hasselt, Belgium, Promoter

Prof. Dr M. Vrijheid, Universitat Pompeu Fabra, Barcelona, Spain, Promoter

Prof. Dr J.Sunyer, Universitat Pompeu Fabra, Barcelona, Spain

Prof. Dr V.Somers, Hasselt University, Hasselt, Belgium

Prof. Dr R. Slama, University Grenoble-Alpes, Grenoble, France

Prof. Dr M. Weijenberg, Maastricht University, Maastricht, The Netherlands

Dr K. Vrijens, Hasselt University, Hasselt, Belgium

Dr L. Casas, Leuven University, Leuven, Belgium

Summary

Although universal and unavoidable, aging does not occur in a uniform way. Aging is a complex phenotype responsive to both environmental and genetic facts and includes both chronic and acute processes. The Developmental Origins of Health and Disease (DOHaD) hypothesis, often called the Barker hypothesis, suggests that adverse influences in the early-life environment shape the future probability of the development of age-related diseases. One of the most hazardous early life environmental risk factors is air pollution.

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. Furthermore we investigated if placental mtDNA content was an intermediate or modulating factor between air pollution exposure and infant growth. To this end, we used data in both newborns and children from 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 specific objectives of this doctoral dissertation were:

1. To investigate the association between gestational air pollution exposure and birth weight and placental mtDNA content and examine the possible mediating role of mtDNA in the association between air pollution and birth weight (chapter 1)
2. To assess the association between gestational air pollution exposure and infant growth and examine the possible mediating role of placental mtDNA content in this association (chapter 3)
3. To investigate whether leukocyte telomere length at 8 years of age is related to early life air pollution exposure (chapter 4)
4. To evaluate the association between maternal and child obesity and telomere length measured in children aged 8 years (chapter 5)

Using data from two independent European birth cohorts: INMA (n = 376; Spain) and ENVIRONAGE (n = 550; Belgium), we examined the potential mediating role of placental mtDNA content in the association between prenatal air pollution exposure and birth weight (**chapter 2**). Changes in mtDNA content may represent a biologically-relevant intermediate outcome in mechanisms linking air pollution and fetal growth restriction. Mitochondria are sensitive to environmental toxicants due to their lack of repair capacity. We showed that a 10 µg/m³ increment in average nitrogen dioxide (NO_2) exposure during pregnancy was associated with a 4.9% decrease in placental mtDNA content and a 48g decrease in birth weight. Higher placental mtDNA content was associated with a higher mean birth weight. Ultimately, in INMA, 10% of the association between prenatal NO_2 exposure and birth weight was mediated by changes in placental mtDNA content.

Among 336 INMA children, we assessed whether prenatal NO_2 exposure was associated with infant growth and whether this association was mediated by growth at birth (length and weight) and/or placental mtDNA content (**chapter 3**). We showed that prenatal NO_2 exposure was inversely associated with several infant growth parameters. These associations seemed to be mediated by birth length and weight. Furthermore, 5.5% of the association between trimester 1 NO_2 exposure and length at 6 months of age could be mediated by placental mtDNA content.

Findings of our study on the association between early life air pollution exposure and telomere length at 8 years are presented in **chapter 4**. Using data of the multi-centre birth cohort study in six European countries HELIX, we found that increased pre- and postnatal exposure to air pollutants lead to shorter leukocyte telomere length in 8 year old children. Additionally, each doubling in residential distance to nearest major road during childhood was associated with a lengthening in leukocyte telomere length.

Chapter 5 focuses on various indices of obesity and their associations with leukocyte telomere length in 8 year old children. Children with mothers with a higher pre-pregnancy BMI had shorter telomeres. Each unit increase in child BMI z-score was associated with significant shorter telomere length. Inverse borderline significant associations were observed between telomere length and a

unit increase in child waist circumference z-score and skinfold thickness z-score. Finally, children with a higher fat mass z-score did not have significant shorter telomere length.

In **chapter 6** we discuss our findings in relation to the available literature, the public health implications of our work, and the limitations of epidemiological research.

Samenvatting

Hoewel veroudering een algemeen en onvermijdelijk proces is, gebeurt het niet via een vast patroon. Veroudering is een complex fenotype dat reageert op zowel omgevings- als genetische factoren en zowel een chronisch als acuut proces omvat. De hypothese van de foetale oorsprong van ziektes op volwassen leeftijd (DOHaD), vaak Barker hypothese genoemd, stelt dat blootstelling aan risicofactoren in het vroege leven de kans verhogen op de ontwikkeling van ouderdom gerelateerde ziektes. Luchtverontreiniging behoort tot één van de meest schadelijke omgevingsblootstellingen.

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. Verder hebben we ook onderzocht of placentaal mtDNA inhoud een tussenliggend of modulerende factor is tussen blootstelling aan luchtverontreiniging en groei van het kind. Om dit te onderzoeken, hebben we data gebruikt van pasgeborenen en kinderen van 3 verschillende geboortecohorten: De Belgische ENVIRONAGE (ENVIRonmental influence ON AGEing in early life) geboortecohort, de Spaanse INMA (INfancia y Medio Ambiente; Environment and Childhood) geboortecohort and de multi-center Europese geboortecohort studie HELIX (Human Early-Life Exposome). De specifieke doelstellingen van dit proefschrift waren:

1. Het onderzoeken van het verband tussen prenatale luchtverontreinigingsblootstelling, geboortegewicht en placentaal mtDNA inhoud en evalueren of placentaal mtDNA inhoud een mogelijke mediator is van het verband tussen prenatale luchtverontreinigingsblootstelling en de groei van het kind (hoofdstuk 2).
2. Het evalueren van het verband tussen prenatale luchtverontreinigings-blootstelling en de groei van het kind en onderzoeken of placentaal mtDNA inhoud een mogelijke mediator is van het verband tussen prenatale luchtverontreinigingsblootstelling en de groei van het kind (hoofdstuk 3).
3. Evalueren of blootstelling aan luchtverontreiniging tijdens het vroege leven verbonden is met leukocyt telomeerlengte op 8 jarige leeftijd (hoofdstuk 4).

4. Het evalueren van het effect van veranderingen in obesitas parameters op telomeerlengte gemeten in 8 jarige kinderen (hoofdstuk 5).

Gebruik makend van gegevens van twee onafhankelijke Europese geboorte cohorts: INMA (n = 376; Spanje) en ENVIR*ON*AGE (n = 550; België), onderzochten we de potentiele mediërende rol van placentaal mtDNA inhoud in het verband tussen prenatale luchtverontreinigingsblootstelling en geboortegewicht (**hoofdstuk 2**). Veranderingen in mitochondriaal DNA inhoud kan een biologisch relevante tussenliggende factor zijn in mechanismen die luchtverontreiniging verbinden met foetale groei restrictie. Mitochondria zijn gevoelig voor milieuverontreinigers omdat ze een onvoldoende herstel capaciteit hebben. In deze studie hebben we aangetoond dat een toename van 10 µg/m³ in stikstofdioxide (NO_2) blootstelling verbonden is met een daling van 4.9% in placentaal mtDNA inhoud en een daling van 48g in geboortegewicht. Hoger placentaal mtDNA inhoud was verbonden met een hoger gemiddeld geboortegewicht. Ten slotte werd 10% van het verband tussen prenataal NO_2 blootstelling en geboortegewicht geïnterfereerd door veranderingen in mtDNA inhoud.

Bij 336 INMA kinderen hebben we onderzocht of prenataal NO_2 blootstelling was verbonden met de groei van het kind en of deze associatie was geïnterfereerd door groei bij de geboorte (geboortegewicht en geboortelengte) en/of placentaal mtDNA inhoud (**hoofdstuk 3**). We hebben aangetoond dat prenatale blootstelling aan NO_2 omgekeerd verbonden was met verschillende groei parameters in het kind. Deze verbanden leken gemedieerd te zijn door geboortegewicht en geboortelengte. Verder bleek 5.5% van het verband tussen blootstelling aan NO_2 tijdens het eerste trimester van de zwangerschap en lengte op 6 jarige leeftijd geïnterfereerd te zijn door placentaal mtDNA inhoud.

Bevindingen van ons onderzoek naar het verband tussen luchtverontreinigings-blootstelling tijdens het vroege leven en telomeerlengte op 8 jarige leeftijd wordt voorgesteld in **hoofdstuk 4**. Gebruik makend van het multi-centrum geboortecohort in zes verschillende Europese landen HELIX, vonden we dat een verhoogde pre- en postnatale blootstelling aan luchtverontreiniging leidt tot kortere telomeren in 8 jaar oude kinderen. Verder blijkt dat een verdubbeling in

de afstand van de weg tot de woonplaats tijdens de kinderjaren is verbonden met een verlenging van telomeerlengte.

Hoofdstuk 5 gaat over verschillende obesitas merkers en hun effect op telomeer lengte in 8 jaar oude kinderen. Kinderen van moeders met een hogere pre-zwangerschap BMI hadden kortere telomeren. Elke eenheid toename in de BMI van het kind was verbonden met significant kortere telomeren. Omgekeerde grensverbondenheid werd geobserveerd tussen telomeerlengte en een eenheid toename in de buikomtrek en huiddikte van het kind. Uiteindelijk vonden we dat kinderen met een hogere vetmassa niet verbonden was met kortere telomeerlengte.

In **hoofdstuk 6** bespreken we onze bevindingen in relatie tot de beschikbare literatuur, de relevantie ervan voor de volksgezondheid en de beperkingen van epidemiologisch onderzoek.

Table of contents

Contents

List of abbreviations

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

CHAPTER 1

General Introduction

Although universal and unavoidable, aging does not occur in a uniform way. The question why some subjects grow old while remaining free from disease whereas others prematurely die remains largely unanswered. Aging is a complex physiological phenomenon responsive to both environmental and genetic factors and includes both chronic and acute processes.[1, 2] 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 an adaptive response in the fetus to in utero exposure could result in persistent changes that influence health in later life.[3] Pro-inflammatory risk factors might influence the aging phenotype through their actions on the primary hallmarks of aging.

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 might be more susceptible to the effects of air pollution exposure compared to adults.[26, 27] 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]. In children, studies have reported an association between air pollution exposure and adverse infant growth. One study in children aged 6-60 months showed that exposure to PM_{10} during pregnancy lowered children's weight and height [44]. In a cohort of children aged 5-11 years traffic-related air pollution during childhood exerted a significant effect on BMI growth [45]. In a rat model, prenatal exposure to ambient air induced inflammatory and lipid oxidation in the lung, which then

spilled over systemically, leading to metabolic dysfunction and weight gain in both rat dams and their 8-week old offspring [46].

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 [47].

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.[48]

2. Obesity

WHO defined obesity according to the ranges of body mass index (BMI = weight [kg]/height [m²]. In adults, 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.[49] In children from 5-19 years, a BMI-for-age value within the range of -2SD and +1SD is categorized as normal, within +1SD and +2SD as overweight and ≥ 2SD as obese.[50] 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.[49, 51] 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.[52]

The prevalence of overweight and obesity is rising worldwide, with the increase in childhood obesity a particular cause for concern.[49] This sharp rise is could be attributed to the high-calorie diet and the sedentary lifestyle adopted recently by many populations.[53, 54] Furthermore, prenatal exposure to certain endocrine disruptors, even at low concentrations, may play a role in the current obesity epidemic.[55]

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.[56]

3. Age related biomarkers

Hallmarks of aging can be grouped into three main categories: genomic instability, telomere attrition, and epigenetic alterations. These are followed by antagonistic responses to such damage: altered mitochondrial function and cellular senescence [57]. In this dissertation we used telomeres and mitochondrial DNA as markers of biological aging.

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.[58] 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.[59] 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.[60-62] 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.[63] 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.[64, 65]

Studies among population show that persons with shorter telomere length in leukocytes have an increased risk for aging-related chronic diseases, such as cancer[66], type 2 diabetes[67], and cardiovascular disease[68]. 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.[69-71] This variation may be the result of both genetic and environmental factors.[72]

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.[73, 74] Telomeres are highly sensitive to oxidative stress, due to their high guanine content and the deficient repair system of single strand breaks.[75] Furthermore, the presence of the unrepaired nucleotides might interface with the replication fork and as such increase telomere shortening.[76] 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.[77-79] Each cell contains approximately 200 to 2,000 mitochondria, each carrying 2-10 copies of mitochondrial DNA (mtDNA).[80] 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.[81]

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.[82] The major differences between human nuclear DNA (nDNA) and mtDNA is that the latter lacks protective histones,

chromatin structure, introns and sufficient DNA repair capacity.[81] 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.[79]

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[83-85], breast cancer[86, 87], multiple sclerosis[88], renal cell carcinoma[89], and cardiovascular illness[90, 91]. Contrary, increases in mtDNA content have been associated with diseases such as pancreatic cancer[92], lung cancer[93] and intrauterine growth restriction in human placenta[94, 95]. 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. [29, 32, 37-39, 45] 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)

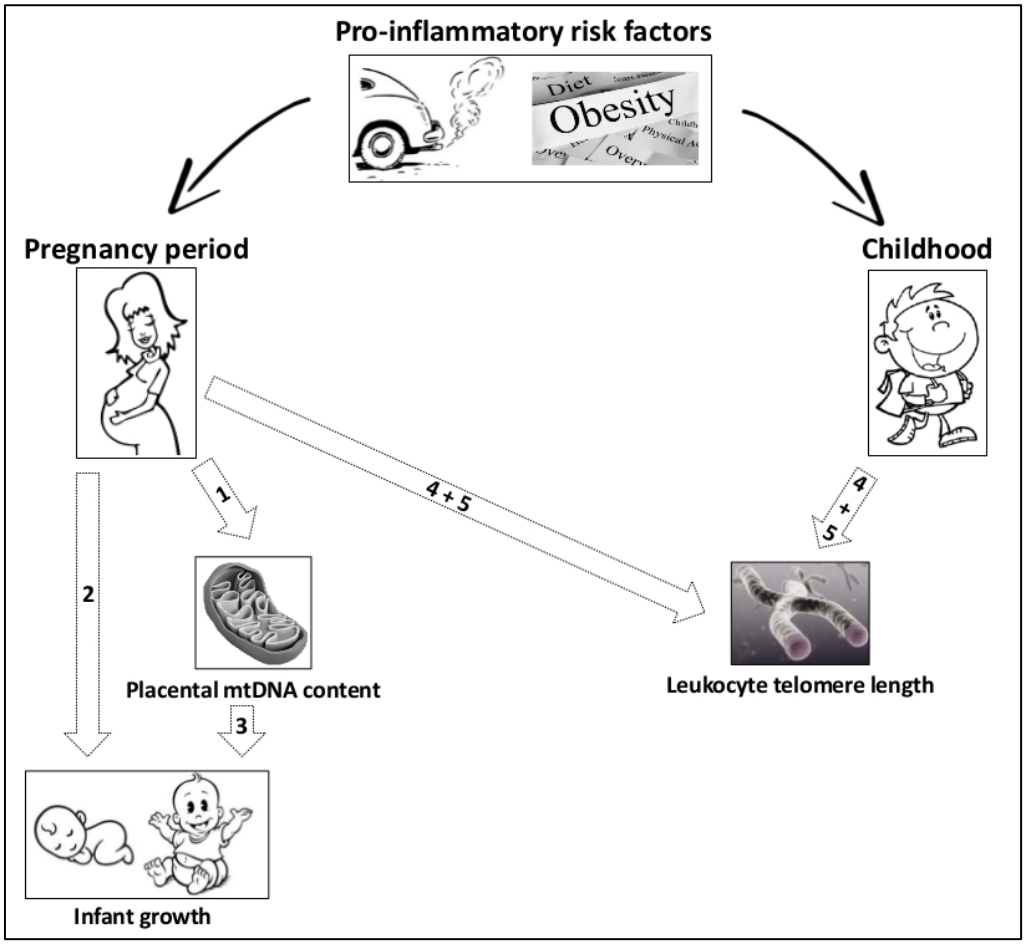

Figure 1. Schematic overview of the objectives of this doctoral dissertation

5. Projects

5.1 ENVIR*ON*AGE (ENVIRonmental influence ON early AGEing)

ENVIR*ON*AGE is a birth cohort in Belgium who was previously described in detail by Janssen *et al* (2017) [96]. Briefly, mother-child pairs are enrolled in the on-going birth cohort when they arrived at the East-Limburg Hospital in Genk (Belgium) for delivery. Participating mothers provided written informed consent for the collection of bio specimens, including placental biopsies and cord blood samples, as well as lifestyle and medical data. In the post-delivery ward the mothers complete study questionnaires to provide detailed information. In **chapter 2**, we used a subset of 556 mother-child pares, for which mtDNA content was measured.

5.2 INMA (INfancia y Medio Ambiente; Environment and Childhood)

INMA is a Spanish population-based birth cohort study that recruited pregnant women in four centers (Valencia, Sabadell, Gipuzkoa, and Asturias), following a common protocol [97]. The birth cohort was described previously by Guxens *et al* (2012) [97]. Briefly, recruitment took place during the first pre-natal visit (10–13 weeks of gestation) in the main public hospital or health centre of each study area. Informed consents were obtained from the mothers for the collection of placental biopsies and cord blood samples. Mothers completed study questionnaires during the different stages of pregnancy to provide detailed information. In **chapter 2**, we used 376 mother-child pairs respectively, for which mtDNA content was measured. In **chapter 3**, we used 336 mother-child pairs respectively, for which mtDNA content was measured. Repeated height and weight measures from birth to 6 months of age were extracted from medical records. Additionally, during the 1-year follow up of INMA child height and weight were measured.

5.3 HELIX (Human Early-Life Exposome)

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 [98], the Étude des

Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) study in France [99], the INfancia y Medio Ambiente (INMA) cohort in Spain [97], the Kaunus cohort (KANC) in Lithuania [100], the Norwegian Mother and Child Cohort Study (MoBa) [101] and the RHEA Mother Child Cohort study in Crete, Greece [102]. This study was described previously by Vrijheid *et al* [103]. All participating women provided informed written consent. In this dissertation we 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 (6-11 years) was organized 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. In **chapter 4** and **chapter 5**, we used 1396 mother-child pairs, for which leukocyte telomere length was measured in the children.

CHAPTER 2

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

Diana B.P. Clemente,[1,2] Maribel Casas,[2,3,4] Nadia Vilahur,[3] Haizea Begiristain,[7] Mariona Bustamente,[2,4,5,6] Anne-Elie Carsin,[2] Mariana F. Fernández,[4,8,9] Frans Fierens,[10] Wilfried Gyselaers,[11] Carmen Iñiguez,[4,12,13] Bram G. Janssen,[1] Wouter Lefebvre,[14] Sabrina Llop,[4,12] Nicolás Olea,[4,8,9] Marie Pedersen,[2,4,15] Nicky Pieters,[1] Loreto Santa Marina,[4,7] Ana Souto,[4,16] Adonina Tardón,[4,16] Charlotte Vanpoucke,[10] Martine Vrijheid,[2,4,5] Jordi Sunyer,[2,4,5,17] and Tim S. Nawrot.[1,18]

[1]Center for Environmental Sciences, Hasselt University, Diepenbeek, Belgium;
[2]Center for Research in Environmental Epidemiology (CREAL), Barcelona, Spain;
[3]Institute for environmental medicine (IMM), Karolinska Institutet, Sweden
[4]CIBER de Epidemiología y Salud Pública (CIBERESP), Institute of Health Carlos III, Madrid, Spain;
5Universitat Pompeu Fabra, Barcelona, Spain;
[6]Center for Genomic Regulation (CRG), Barcelona, Spain;
[7]Health Research Institute (BIODONOSTIA), Gipuzkoa, Spain;
[8]Department of Radiology, University of Granada, Granada, Spain;
[9]Instituto de Investigación Biosanitaria de Granada, Granada, Spain;
[10]Belgian Interregional Environment Agency, Brussels, Belgium;
[11]Department of Obstetrics, East-Limburg Hospital, Genk, Belgium;
[12]FISABIO, Valencia, Spain;
[13]University of Valencia, Valencia, Spain;
[14]Flemish Institute for Technological Research (VITO), Mol, Belgium;
[15]INSERM (National Institute of Health and Medical Research), Grenoble, France;
[16]Molecular Epidemiology of Cancer Unit, University Institute of Oncology, Oviedo, Spain;
[17]IMIM (Hospital del Mar Research Institute), Barcelona, Spain;
[18]Department of Public Health & Primary Care, Leuven University (KU Leuven), Leuven, Belgium

Environmental Health Perspective 2016;124:5

Abstract

Background Mitochondria are sensitive to environmental toxicants due to their lack of repair capacity. Changes in mitochondrial DNA (mtDNA) content may represent a biologically-relevant intermediate outcome in mechanisms linking air pollution and fetal growth restriction.

Objective We investigated whether placental mtDNA content is a possible mediator of the association between prenatal NO2 exposure and birth weight.

Methods We used data from two independent European cohorts: INMA (n=376; Spain) and ENVIR*ON*AGE (n=550; Belgium). Relative placental mtDNA content was determined as the ratio of two mitochondrial genes (*MT-ND1* and *MTF3212/R3319*) to two control genes (*RPLP0* and *ACTB*). Effect estimates for individual cohorts and the pooled dataset were calculated using multiple linear regression and mixed models. We also performed a mediation analysis.

Results Pooled estimates indicated that a 10µg/m^3 increment in average NO_2 exposure during pregnancy was associated with a 4.9% decrease in placental mtDNA content (95% confidence interval (CI): -9.3, -0.3%). and a 48g decrease (95% CI: -87, -9g) in birth weight. However, the association with birth weight was significant for INMA (-66g; 95% CI: -111, -23g) but not for ENVIR*ON*AGE (-20g; 95% CI: -101, 62g). Placental mtDNA content was associated with significantly higher mean birth weight (pooled analysis, IQR increase: 140g; 95% CI: 43, 237g). Mediation analysis estimates, which were derived for the INMA cohort only, suggested that 10% (95% CI: 6.6, 13.0g) of the association between prenatal NO_2 and birth weight was mediated by changes in placental mtDNA content.

Conclusions Our results suggest that mtDNA content can be one of the potential mediators of the association between prenatal air pollution exposure and birth weight.

Introduction

In recent years, traffic related air pollution has been considered an important risk factor for adverse reproductive health effects. Prenatal exposure to nitrogen dioxide (NO_2) has been associated with low birth weight, intrauterine growth restriction, and preterm birth [29, 30]. Infants with low birth weight are at higher risk of mortality and morbidity, and impaired cognitive development compared to infants with higher birth weight [104, 105].

Mitochondria are intracellular organelles that are essential for the aerobic production of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS). These “power plants of the cell” also play a critical role in signaling transduction for cell proliferation, apoptosis, calcium storage and metabolism [77-79]. Several studies have identified the generation of oxidative stress, by producing reactive oxygen species (ROS), as one of the major mechanisms by which air pollution exert adverse biological effects [106, 107]. Mitochondria are the major intracellular sources of ROS, which are generated under normal conditions as by-product of OXPHOS [82]. On the other hand, mitochondria are also the primary targets of oxidative stress because in comparison with nuclear DNA (nDNA), mitochondrial DNA (mtDNA) is lacking the protective strategies associated with nDNA, such as protective histones, chromatin structure, and sufficient DNA repair capacity [81]. Consequently, mtDNA is particularly vulnerable to ROS-induced damage and has a high mutation rate [78]. Mitochondria compensate for these mutations by increasing their number and their replication rate resulting in a change in mtDNA content, which therefore reflects mitochondrial damage and dysfunction [77, 78, 82, 108].

The placenta plays a unique role in gases, nutrient and waste transfer between the mother and developing child. It is both a metabolic and an endocrine organ. However, the placenta has a limited capability to metabolize a large number of foreign compounds [109]. The placenta requires energy to maintain its function and this energy provision is regulated by mitochondrial function of placenta cells [110]. Air pollution exposure is hypothesized to affect the fetus directly through trans placental exposure or indirectly by affecting maternal health and body functions [111]. This could impair the placental exchange of nutrients and gases. Under poor nutritional conditions the fetus can adapt its mitochondrial structure and

metabolism. Therefore, this "metabolic reprogramming" could be at the origin of adverse birth outcomes [112].

Recently, it has been shown that exposure to particulate air pollution during pregnancy was associated with placental mtDNA content [113]. We hypothesized that changes in mtDNA content may represent a biological causal effect along the path linking air pollution exposure with the potential adverse health effects of the offspring. Based on two independent European birth cohorts (INMA and ENVIR*ON*AGE), we aimed to assess the role of mediating effects of placental mtDNA content on the association of prenatal NO_2 exposure with birth weight.

Methods

Study design and population

The Spanish population-based birth cohort study INMA (INfancia y Medio Ambiente; Environment and Childhood) recruited pregnant women in four centers (Valencia, Sabadell, Gipuzkoa, and Asturias), following a common protocol [114]. A total of 2616 pregnant women were enrolled between 2004 and 2008 during the first trimester of pregnancy at public primary health care centers or public hospitals if they fulfilled the inclusion criteria: ≥ 16 years of age, a singleton pregnancy, intention to deliver at the reference hospital, no problems of communication, and no assisted conception. Of all eligible pregnant women, 57% agreed to participate. The present analysis included 390 mother-newborn pairs from the INMA cohort with placental mtDNA content data. A comparison of this INMA subcohort with the whole INMA cohort (n=2,616) did not show differences in maternal age, pre-gestational BMI, parity, and ethnicity.

In the population-based birth cohort study ENVIR*ON*AGE (ENVIRonmental influence ON AGEing), 556 pregnant women were enrolled between 2010 and 2013 at the South-East-Limburg Hospital in Genk (Belgium) when they arrived for delivery. The inclusion criteria were: ≥ 18 years of age, singleton pregnancy, and to be able to fill out questionnaires in Dutch. The overall participation rate of eligible mothers was 56%. A comparison of the ENVIRONAGE birth cohort with all births in Flanders [115] did not show differences in maternal age, pre-gestational BMI, parity, and ethnicity.

Study approval was obtained from the ethics committees of each participating center and informed consents were obtained from the mothers. In both cohorts information on maternal age, ethnicity, maternal smoking status, place of residence, pre-pregnancy body mass index (BMI), and parity was obtained. In INMA they were obtained by questionnaires and interviews, in ENVIR*ON*AGE by questionnaires. Perinatal parameters such as newborn's sex, birth date, birth weight, gestational age, and delivery by caesarean section were collected by birth records. Information about maternal and child characteristics were standardized to perform a harmonized, pooled analysis.

Sample collection

In the INMA cohort, placentas were randomly collected in approximately one out of four deliveries (n=502). The entire placentas were frozen after delivery at -20°C until they were transferred on dry ice to the Hospital Universitario San Cecilio (HUSC) Biobank in Granada (Spain) and stored at -86°C. No information was available about the time between delivery and storage at the different Spanish hospitals involved in the study. MtDNA content was measured in 390 out of the 502 randomly selected placentas in INMA. In the ENVIR*ON*AGE cohort, all placentas (n=556) were collected after delivery and were frozen within 10 min at -20°C. In both cohorts, placentas were thawed minimally to obtain tissue biopsies for DNA extraction. To minimize the impact of within-placental variability, biopsies were all taken 1-1.5 cm below the chorio amniotic membrane at a fixed location and preserved at -80°C [113].

DNA extraction and measurement of mtDNA content

In the INMA cohort, DNA was extracted from placental tissue cells using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, United States) following the manufacturer's instructions. In the ENVIR*ON*AGE cohort, DNA was extracted from placental tissue cells using the QIAamp DNA minikit (Qiagen, Inc., Venlo, Netherlands) following the manufacturer's instructions. In both cohorts, DNA samples were quantified using the Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, Netherlands) and the Quant-iT™ PicoGreen®

dsDNA Assay Kit (Life Technologies, Foster City, CA, United States) using the Omega Fluostar plate reader (BMG LABTECH, Ortenberg, Germany).

MtDNA content was measured in placental tissue cells by determining the ratio of two mitochondrial genes (mitochondrial encoded NADH dehydrogenase subunit 1 (*MT-ND1*) and mitochondrial forward primer for nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*)) to two nuclear control genes (acidic ribosomal phosphoprotein P0 (*RPLP0*), and beta-actin (*ACTB*)) using a quantitative real-time polymerase chain reaction (qPCR) assay [113]. qPCR was performed using 2.5 µl of extracted DNA (5 ng/µl) and 7.5 µl of master mix consisting of Fast SYBR® Green I dye 2x (Life Technologies; 5 µl/reaction), forward (0.3 µl/reaction – 300 nM) and reverse (0.3 µl/reaction – 300 nM) primer (Biolegio, Nijmegen, Netherlands) and RNase free water (1.9 µl/reaction). Samples were run in triplicate in 384-well format. qPCR was performed using the 7900HT Fast Real-Time PCR System (Life Technologies, Foster City, CA, United States) with following thermal cycling profile: 20 sec at 95°C (activation), followed by 40 cycles of 1 sec at 95°C (denaturation) and 20 sec at 60°C (annealing/extension). At the end of each run a melting curve analysis was performed to confirm amplification specificity and absence of primer dimers (15 sec at 19°C, 15 sec at 60°C, 15 sec at 95°C). qBase software (Biogazelle, Zwijnaarde, Belgium) was used to normalize data and correct for run-to-run differences [113].

Ambient air pollution assessment

In INMA, ambient concentrations of NO_2 were measured with the aid of passive samplers (Radiello, Fundazione Salvatore Maugeri, Padua, Italy) distributed outside across the study areas according to geographic criteria, taking into account the expected pollution gradients and the distribution of the residences of the participating women. The samplers remained exposed during various seven day sampling periods through pregnancy. The methodology has been described in detail elsewhere [116, 117] and further sampling information is given in Supplemental Material, Table S1. Temporally adjusted land use regression (LUR) models were used to predict NO_2 levels at women's residential addresses and taking into account residential changes if women lived at least 2 months of

pregnancy in the new residence. To calculate individual exposure during pregnancy, annual average NO_2 estimates from the LUR were temporally adjusted using serial records from the network of monitoring stations covering each of the four INMA study areas. The validation statistics gave a spatial explained variance (R^2) for annual mean NO_2 from 0.52 to 0.75 in the four INMA subcohorts (See Supplemental Material, Table S1).

In ENVIR*ON*AGE regional background levels of NO_2 for each women's home address were calculated using a kriging interpolation method [118, 119] that uses land cover data obtained from satellite images combined with a dispersion model [120]. This model chain provided NO_2 values, combining data from the Belgian telemetric air quality network, point sources and line sources, which was then interpolated to a high resolution receptor grid. This was used to obtain NO_2 levels at women's residential addresses, taking into account any residential change during pregnancy. The validation statistics gave a temporal explained variance (R^2) for hourly averages > 0.80 and a spatial explained variance (R^2) for annual mean NO_2 of 0.82.

In order to explore potentially critical exposures during pregnancy, individual NO_2 concentrations were calculated for the three trimesters of pregnancy using the same procedure utilized for the entire pregnancy: 1–13 weeks starting from date of conception (Trimester 1), 14–28 weeks (Trimester 2) and 29 weeks to delivery (Trimester 3).

More details on exposure measurements for INMA and ENVIR*ON*AGE can be found in Supplemental Materials, Table S1.

Statistical analysis

Continuous data were checked for normality using the Shapiro-Wilk test statistic. Placental mtDNA content data was right skewed and therefore logarithmically transformed (log10). Generalized additive models (GAMs) were used to assess the linearity of the associations between (i) prenatal NO_2 exposure and mtDNA content, (ii) mtDNA content and birth weight, and (iii) prenatal NO_2 exposure and birth weight (See Supplemental Materials, Figure S1). Multiple linear regression models were used in ENVIR*ON*AGE. Multiple linear mixed models with

a random cohort effect were used in INMA and in the pooled dataset (4 INMA cohorts and ENVIR*ON*AGE). Covariates used in the model were gestational age (linear and quadratic term), newborn's sex, maternal age, ethnicity, parity, smoking status, education, season of birth (January-March, April-June, July-September, October-December), and pre-pregnancy maternal BMI. For the present analysis, we excluded 14 mother-newborn pairs from INMA and 6 mother-newborn pairs from ENVIR*ON*AGE with missing values in the outcome, exposure and confounders. After these exclusions, the final study population consisted of 376 subjects for INMA and 550 subjects for ENVIR*ON*AGE.

To determine whether placental mtDNA content is a potential mediator of the association between prenatal NO_2 exposure and birth weight we performed a mediation analysis. The direct effect (DE), indirect effect (IE) and total effect (TE) were estimated using the SAS macro developed by Valeri and VanderWeele [121]. The DE represents the effect of prenatal NO_2 exposure on birth weight after controlling for mtDNA content, and the IE is the estimated effect of NO_2 exposure during pregnancy operating through mtDNA content [121]. The proportion of mediation by placental mtDNA content was calculated as the ratio of IE to TE.

A sensitivity analysis was performed, in which all non-vaginal deliveries were excluded, since it has been suggested that fetus could be exposed to different levels of oxidative stress depending on the type of delivery [122]. In another sensitivity analysis we used cohort as a fixed effect instead of a random effect. Further, we tested the interaction between mtDNA content and sex on birth weight by including its interaction term in the full model. In INMA we also performed an additional sensitivity analysis taking into account the time-activity patterns of the women during pregnancy. Because it has been indicated that time-activity patterns during pregnancy should be considered to improve the accuracy of exposure measurement and reduce exposure misclassification [123], we calculated the time spent at home from self-reported information (questionnaire at week 32) and restricted in INMA our analysis to women who spent ≥ 15 hours/day at home [123]. This information was not available for the ENVIR*ON*AGE cohort.

All statistical analysis were conducted using SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA).

Results

Characteristics of the study population

Characteristics of the 376 and 550 mother-newborn pairs in respectively the INMA and the ENVIRONAGE cohort are shown in table 1. INMA mothers were more likely to be primiparous, older, lower educated, of European origin and had lower BMI compared with mothers from ENVIRONAGE. The mean birth weight was lower and placental mtDNA content higher in INMA newborns compared with newborns from ENVIRONAGE.

The mean (±SD) pregnancy average exposure to NO_2 was 25.5 ± 11.4 µg/m³ and 21.1 ± 4.2 µg/m³ in INMA and ENVIRONAGE, respectively. Similar differences in exposure levels between cohorts were observed in the mean trimester concentrations (Table 2).

Association between placental mtDNA content and NO_2 exposure

In the INMA cohort, NO_2 exposure during each trimester and the entire pregnancy was negatively and significantly associated with placental mtDNA content (Table 3). These results were consistent in the direction of the associations in the 4 different INMA subcohorts (See Supplemental Materials, Table S2). Each 10 µg/m^3 increment in average pregnancy exposure was associated with a lower placental mtDNA content of 5.5% (95% Confidence Interval (CI): -8.8, -2.1%). In the ENVIRONAGE cohort, NO_2 exposure was also negatively associated with placental mtDNA content in each trimesters and in the entire pregnancy, but it was only statistically significant during the second (-11.1%, 95% CI: -19.9, -1.2%) and third trimester of pregnancy (-13.5%, 95% CI: -20.1, -6.4%) (Table 3). The pooled analysis showed a statistically significant association with exposure during the second and third trimesters as well as in the entire pregnancy (Table 3).

Table 1. Characteristics of INMA and ENVIR*ON*AGE participants

Characteristics	INMA (n=376)	ENVIR*ON*AGE (n=550)
Maternal		
Age, years*	32.2(± 3.9)	29.0 (± 4.6)
Smoking*		
Never	170 (45.2)	354 (64.4)
Quit smoking before week 12	143 (38.0)	119 (21.6)
During entire pregnancy	63 (16.8)	77 (14.0)
Education*		
Primary school or none	75 (20.0)	67 (12.2)
Secondary school	167 (44.4)	203 (36.9)
University	134 (35.6)	280 (50.9)
Parity		
1	212 (56.4)	299 (52.4)
2	138 (36.7)	195 (35.5)
≥3	26 (6.9)	56 (10.2)
Pre-pregnancy BMI, kg/m^2	23.5 (± 4.4)	24.1 (± 4.5)
Ethnicity		
European	343 (91.2)	485 (88.2)
Non-European	33 (8.8)	65 (11.8)
Cohort		
Valencia	63 (16.8)	/
Asturias	37 (9.8)	/
Sabadell	120 (31.9)	/
Gipuzkoa	156 (41.5)	/
ENVIR*ON*AGE	/	550 (100.0)
Time spent at home		
> 15 hours a day	214 (56.9)	/
≤ 15 hours a day	162 (43.1)	/
Newborn		
Gestational age, weeks	39.9 (± 1.3)	39.3 (± 1.2)
Sex		
Male	194 (51.6)	277 (50.4)
Female	182 (48.4)	273 (49.4)
Season at birth		
January-March	99 (26.3)	156 (28.4)
April-June	102 (27.1)	131 (23.8)
July-September	92 (24.5)	143 (26.0)
October-December	83 (22.1)	120 (21.8)
Preterm delivery (<37 weeks)		
Yes	7 (1.9)	14 (2.6)
No	369 (98.1)	536 (97.5)
Vaginal delivery		
Yes	322 (85.6)	521 (94.7)
No	54 (14.4)	29 (5.3)
Birth weight, g*	3,290 (±423)	3,429.6 (±432)
Placental mtDNA content*	1.3 (1.1-1.5)	1.0 (0.7-1.4)

Continuous covariates expressed by mean and standard deviation (SD) or geometric mean and 25– 75th percentile; categorical covariates described by frequencies (%). Differences between cohorts were assessed using independent t-tests. Subjects without available information have been excluded before performing the independent t-tests. *P value < 0.05.

Table 2. Descriptive statistics of prenatal NO_2 exposure ($\mu g/m^3$)

NO_2 exposure ($\mu g/m^3$)	Mean ± SD	P5	P25	P50	P75	P95	r[a]
INMA (n=376)							
Trimester 1	26.1 ± 12.9	5.6	16.4	23.1	33.7	74.2	0.91*
Trimester 2	25.6 ± 11.6	5.7	16.4	24.8	31.2	74.7	0.93*
Trimester 3	25.7 ± 12.1	5.7	16.9	23.8	32.3	74.4	0.92*
Entire pregnancy	25.5 ± 11.4	5.7	17.2	24.0	32.3	66.7	-
ENVIRONAGE (n=550)							
Trimester 1	20.7 ± 6.1	7.3	16.3	20.2	24.9	39.2	0.61*
Trimester 2	20.8 ± 6.0	8.6	16.2	20.5	25.1	46.0	0.86*
Trimester 3	21.4 ± 6.1	9.2	16.9	20.8	25.6	40.3	0.66*
Entire pregnancy	21.1 ± 4.2	12.6	18.2	20.8	23.7	40.3	-
INMA + ENVIRONAGE (n=926)							
Trimester 1	22.7 ± 9.8	5.6	16.1	21.2	26.8	74.2	0.86*
Trimester 2	22.6 ± 9.1	5.7	15.9	21.3	27.3	74.7	0.91*
Trimester 3	23.0 ± 9.3	5.7	16.7	21.5	27.3	74.4	0.88*
Entire pregnancy	22.7 ± 8.3	5.7	17.6	21.2	25.6	66.7	-

Continuous covariates expressed by mean and standard deviation (SD)

[a]Pearson correlation between the pregnancy average and trimester specific exposures

*P-value < 0.001

Association between birth weight and NO_2 exposure

The association between birth weight and prenatal NO_2 exposure was significant in the INMA cohort for all three trimesters of pregnancy (Table 4). Each 10 $\mu g/m^3$ increment in average pregnancy NO_2 exposure was associated with a 66.4g (95% CI: -111.0, -22.7) decrease in birth weight (Table 4). These results were consistent in the direction of the associations in the 4 different INMA subcohorts (See Supplemental Materials, Table S3). In the ENVIRONAGE cohort, estimates were in the same direction although the estimated effects were smaller than in INMA and did not reach significance (-19.8g; 95% CI: -101.1, 61.7). After pooling both cohorts, the association between birth weight and NO_2 was significant in all pregnancy trimesters and in the entire pregnancy (-47.5g, 95% CI: -86.6, -8.5) (Table 4).

Table 3. Percent change in placental mtDNA content in association with prenatal NO_2 exposure in INMA, ENVIRONAGE, and in the pooled sample

Pregnancy period	Differences in placental mtDNA content (%)	95% CI	P-value
INMA (n=376)[a,b]			
Trimester 1	-4.1	-7.1, -1.1	0.007
Trimester 2	-5.0	-8.0, -2.0	0.002
Trimester 3	-4.9	-7.9, -1.8	0.003
Entire pregnancy	-5.5	-8.8, -2.1	0.002
ENVIRONAGE (n=550)			
Trimester 1	-5.1	-15.5, 6.6	0.38
Trimester 2	-11.1	-19.9, -1.24	0.03
Trimester 3	-13.5	-20.1, -6.4	0.003
Entire pregnancy	-10.1	-20.1, 1.24	0.08
INMA + ENVIRONAGE (n=926)[c]			
Trimester 1	-2.5	-6.4, 1.6	0.22
Trimester 2	-4.4	-8.4, -0.3	0.04
Trimester 3	-5.2	-9.1, -1.2	0.01
Entire pregnancy	-4.9	-9.3, -0.3	0.04

Effect size was estimated for each 10 µg/m3 increment in exposure to NO2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, maternal smoking status, gestational age (linear and quadratic), pre-pregnancy BMI, parity, ethnicity, season of birth, and education;

[a]Results followed the same direction in all 4 INMA subcohorts (See Supplemental Materials, Table S2).

[b]Four INMA subcohorts were included as random effect.

[c]Cohorts were included as random effect.

Association between placental mtDNA content and birth weight

Placental mtDNA content was positively and significantly associated with birth weight in both cohorts and in the pooled analysis (Table 5). Interaction test show that the interaction of mtDNA content with sex was significant for the individual cohorts and the pooled analysis. This suggests evidence of effect modification by sex. In the pooled analysis, an interquartile range (IQR) increase in mtDNA content was associated with 66 g (95% CI: 18, 114) increase in mean birth weight in boys, compared with 26 g (95% CI: -67,15) in girls (interaction p-value = 0.009)(Table 5).

Table 4. Association between prenatal NO_2 exposure and birth weight in INMA, ENVIRONAGE, and in the pooled sample

Pregnancy period	Differences in birth weight (g)	95% CI	P-value
INMA (n=376)[a,b]			
Trimester 1	-56.2	-94.5, -17.8	0.004
Trimester 2	-56.3	-96.2, -16.4	0.006
Trimester 3	-52.1	-93.8, -12.5	0.01
Entire pregnancy	-66.4	-111.0, -22.7	0.004
ENVIRONAGE (n=550)			
Trimester 1	-20.0	-91.3, 51.3	0.58
Trimester 2	-3.4	-76.4, 69.5	0.93
Trimester 3	-29.9	-98.2, 38.3	0.39
Entire pregnancy	-19.8	-101.1, 61.7	0.63
INMA + ENVIRONAGE (n=926)[c]			
Trimester 1	-44.1	-77.4, -10.8	0.01
Trimester 2	-36.2	-70.9, -1.6	0.04
Trimester 3	-37.5	-71.4, -3.6	0.03
Entire pregnancy	-47.5	-86.6, -8.5	0.02

Effect size was estimated for each 10µg/m3 increment in exposure to NO2 at each mother's residence during the corresponding period.

Models were adjusted for newborn's sex, season of birth, maternal age, maternal smoking status, parity, ethnicity, education, gestational age (linear and quadratic) and pre-pregnancy BMI.

[a]Results followed the same direction in all 4 INMA subcohorts. (See Supplemental Materials, Table S3).

[b]Four INMA subcohorts were included as random effect.

[c]Cohorts were included as random effect.

Table 5. Association between placental mtDNA content and birth weight (g) in INMA, EVIRONAGE, and in the pooled sample

	INMA[a,b]					ENVIRO*N*AGE					INMA + ENVIRO*N*AGE[c]				
	n	**Differences in birth weight (g)**	**95% CI**	**P-value**	**Interaction P-value**	**n**	**Differences in birth weight (g)**	**95% CI**	**P-value**	**Interaction P-value**	**n**	**Differences in birth weight (g)**	**95% CI**	**P-value**	**Interaction P-Value**
All	376	249.0	83.6, 414.3	0.003	0.003	550	129.2	7.8, 259.0	0.04	0.04	926	140.2	43.2, 237.2	0.005	0.009
Boys	194	124.0	45.6, 202.5	0.002	N/A	277	34.0	-34.4, 102.4	0.33	N/A	471	65.9	17.9, 114.0	0.007	N/A
Girls	182	-2.44	-80.5, 75.6	0.95	N/A	273	-15.2	-69.3, 39.0	0.58	N/A	455	26.4	-67.4, 14.6	0.21	N/A

Effect size was estimated for each IQR increment (INMA=0.58; ENVIR*ON*AGE=0.77; Pooled sample=0.76) in mtDNA content; CI: Confidence interval; g: gram; N/A: not applicable

Models were adjusted for gestational age (linear and quadratic), newborn's sex, maternal age, maternal smoking status, pre-pregnancy BMI, parity, ethnicity, season of birth, education, and interaction term sex and mtDNA content.

[b]Four INMA subcohorts were included as random effect.

[c]Cohorts were included as random effect.

Mediation analysis

In INMA, the mediation analysis suggested that 10% (95% CI: 6.6, 13.0) of the association between birth weight and pregnancy average NO_2 exposure may be mediated by differences in placental mtDNA content (Figure 1A). The corresponding estimates for mediation of associations between birth weight and NO_2 exposure during the first, second and third trimester, were 9.1% (95% CI: 5.3, 12.6), 11.4% (95% CI: 7.3, 15.2), and 12.2% (95% CI: 7.7, 16.3), respectively. When we limited the mediation analysis to boys in the INMA cohort, the analysis suggested a mediation effect of 16% (95% CI: 18.7, 13.2) (Figure 1B). Because in the ENVIRONAGE cohort prenatal NO_2 exposure was not significantly associated with birth weight, we did not perform the subsequent mediation analysis. After pooling both cohorts, the estimated proportion of mediation by mtDNA content was not significant for the association between birth weight and pregnancy average NO_2 exposure (2.2%, 95% CI: -2.0, 6.1), or for NO_2 exposure during the different trimesters (See Supplemental Materials, Table S4). Mediation analysis of the pooled data for boys suggested placental mtDNA content mediated 6.4% (95% CI: 2.4, 10.0) of the association between NO_2 during trimester 3 and birth weight, but mediation was not statistically significant for average pregnancy NO_2 or NO_2 during trimester 1 or 2 (See Supplemental Materials, Table S4).

Sensitivity analysis

The reported associations did not change after excluding mother-newborn pairs with non-vaginal deliveries (n=83) (data not shown). Furthermore, when we used cohort as a fixed effect, our reported estimates did not change either (data not shown). Finally, associations were stronger when the analysis was limited to 214 INMA participants who spent ≥ 15 hours/day at home, including associations between NO_2 and birth weight (e.g., for average pregnancy NO_2: 84g; 95% CI: -142, -26 compared with -66g; 95% CI: -111, -23) (See Supplemental Material, Table S5 and Table S6, respectively).

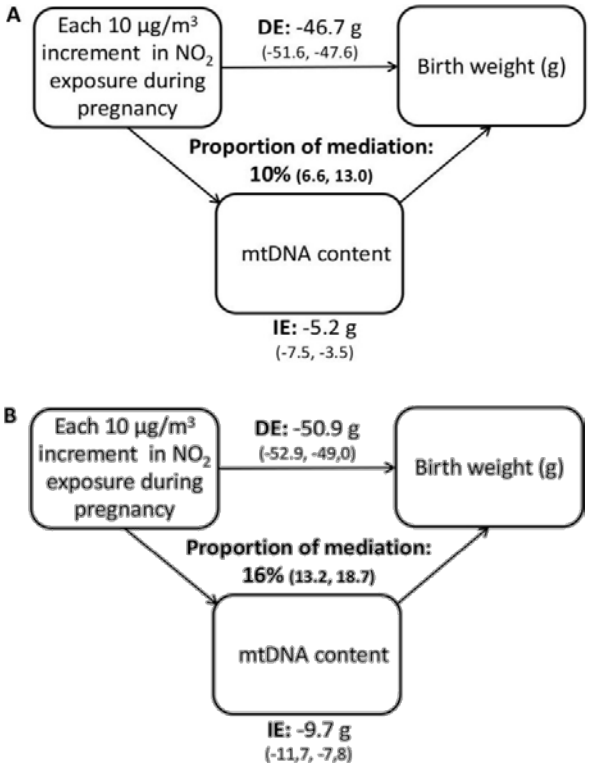

Figure 1. Mediation analysis of the estimated effect of prenatal NO_2 exposure (µg/m3) on birth weight through placental mtDNA content in the INMA cohort. 95% Confidence intervals are given between brackets. A: Whole INMA; B: INMA-boys. Results from mediation analysis with exposure to NO_2 during the entire pregnancy were obtained using the SAS macro developed by Valeri and VanderWeele (Valeri and VanderWeele 2013).. The figure shows placental mtDNA as a potential mediator, the estimates of indirect effect (IE), the estimates of the direct effect (DE) and proportion of mediation. Model was adjusted for gestational age (linear and quadratic term), newborn's sex, maternal age, maternal smoking status, pre-pregnancy BMI, parity, ethnicity, season, education, and INMA subcohort.

Discussion

Mitochondria, the energy producers of the cells, are particularly sensitive to environmental toxicants due to their lack of repair capacity [5]. Fetuses adapt their mitochondrial structure and metabolism when the supply of nutrients is limited [16]. We hypothesized that mitochondrial damage may be a causal

intermediate in biological mechanisms linking air pollution to birth outcomes. In the current study, we evaluated placental mtDNA content, a proxy of mitochondrial damage, as a potential mediator of the association between reduced birth weight and prenatal NO_2 exposure. The key findings, based on two independent European cohorts were: (i) prenatal NO_2 exposure is inversely associated with placental mtDNA content; (ii) placental mtDNA content is positively associated with birth weight; (iii) prenatal NO_2 exposure is inversely associated with birth weight; and (iv) placental mtDNA content can be a potential mediator of the association between birth weight and prenatal NO_2 exposure.

Ambient air contains a mixture of pollutants. NO_2 is frequently used as a surrogate for traffic-related air pollution because it is considered to be a good proxy of other pollutants originating from the same sources [28]. An appreciable number of epidemiologic studies have shown an association between fetal growth restriction and prenatal exposure to air pollution with evidence of statistical significant heterogeneity in the estimated effects among different locations [2, 29]. An earlier observation on the full INMA cohort (n=2,337) reported an estimated decrease in birth weight of 11 g for every 10 $\mu g/m^3$ increment in NO_2 [27]. Data from 14 European mother-child cohorts, including INMA, reported a very weak association between birth weight of 1 g (95% CI: -6, 4 g) and NO_2 during pregnancy [1]. In our current study we estimated a 48 g reduction in birth weight (95% CI: -87, -9) with a 10 $\mu g/m^3$ increment in pregnancy average NO_2 based on the pooled analysis, with a stronger association in the INMA cohort (-66g; 95% CI: -111, -23) then in the ENVIR*ON*AGE cohort (-20g; 95% CI: -101, 62). This association also varied among the four INMA sub-cohorts ranging from -12g (95% CI: -20, 12; n=63) for the Valencia sub-cohort to -156g (95% CI: -378, 67; n=37) for the Asturias sub-cohort (See Supplemental Materials, Table S4). Differences in effect-sizes across the studied cohorts might be due to different levels of exposure, population variability, and variation in meteorological conditions, but also non-causal mechanisms could explain these differences; e.g. differences in study design and conduct, exposure assessment, and differences in residual confounding. Nevertheless, our GAM plots showed linear associations between NO_2 and mtDNA content, NO_2 and birth weight, and

mtDNA content and birth weight in both cohorts (See Supplemental Materials, Figure S1).

We need to consider that our exposure assessment was limited to the residential address of the mothers, and exposure to other air pollutants (e.g. particulate matter and carbon monoxide), environmental and dietary contaminants that have been associated with lower birth weight as well as exposure to NO_2 during commute, at work and elsewhere was not taken into account. Time spent at home can influence associations between ambient air pollution measured at the mother's residence and birth weight [27], in this regard we also found stronger associations within INMA for mothers who spend more than 15 hours per day at home.

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, alter placental growth, decrease placental exchange of nutrients and gases, endocrine disruption, or cause maternal health effects—all of which could possibly lead to altered fetal growth [30]. Oxidative stress-induced DNA damage appears to be a particularly important mechanism of action of urban air pollutants [31]. MtDNA is particularly vulnerable to ROS-induced damage and has been described as a proxy of air pollution induced damage [10, 32]. In this study, we estimated that 10% (95% CI: 6.6, 13.0%) of low birth weight caused by prenatal NO_2 exposure could be explained by placental mtDNA content. This finding was demonstrated in a subsample of 376 mother-child pairs of the INMA cohort. We are aware that epidemiological studies can only show associations, but cannot prove causality; nevertheless, our formal mediation analysis is based on predefined hypothesis and is in line with experimental evidence.

Mechanism through which prenatal exposure to traffic related air pollution might cause placental inflammation and oxidative stress are unclear. The maternal and fetal circulation are separated by the placental barrier that is formed by the syncytiotrophoblast layer, which faces the maternal environment [33]. This barrier contains placental transporters that can block or facilitate foreign compounds [14, 33]. Further, it has been reported that air pollution was associated with increased white blood cells in chronic obstructive pulmonary disease (COPD) patients,

suggesting that air pollutants can elicit systemic inflammation [34]. In addition, it has been observed that human plasma collected from individuals exposed to diesel exhaust for only short periods of time (1 hour) is pro-inflammatory to endothelial cells *in vitro* [35], implying that soluble, pro-inflammatory mediators circulate in the blood after inhalation of diesel. From these studies it might be hypothesized that maternal circulating pro-inflammatory mediators are responsible for associations of prenatal NO_2 with placental mtDNA content and birth weight in our study population. Other mechanisms might include transient receptor potential (TRP) channels which are highly expressed in placenta, and their activation has been suggested to play important roles in placental development and regulating the feto-maternal interface in mice models [36]. If air pollution exposure can result in systemic activation of TRP channels, we might speculate that placental TRP channels are also activated and may mediate our observed effects. In this context, it has been shown recently that TRP channels interacts with a large number of mitochondrial proteins [37].

Induction of ROS levels stimulates autophagy and mitophagy as exemplified by lower mtDNA content in placental tissue [38, 39]. In the current study, stratified analyses indicated a stronger inverse association between placental mtDNA content and prenatal NO_2 exposure in newborn boys than in girls. Indeed sex-dependent susceptibility to oxidative stress has been shown and the antioxidant defenses are apparently different in XX and XY cells [39].

Our study has some limitations. Ambient exposure to air pollution does not account for indoor exposure, which has also been associated with reduced birth weight [40]. Although our results were consistent after multiple adjustments, some residual confounding by some unknown factors that are associated with ambient air pollution, mitochondrial function and mitochondrial DNA content, and birth weight cannot be excluded.

The major strengths of this study are that we tested the different windows of exposure, and made use of two independent birth cohorts in Southern (Spain) and Western Europe (Belgium). Also, we used new methods and their assumptions to study causal interference [25]. Nevertheless, this mediation analysis gives only estimates of the DE, IE and TE. Furthermore, our results of the association between prenatal air pollution exposure and mtDNA content are

in line with those of Janssen et al. [17] in the same birth cohort (ENVIR*ON*AGE) with a smaller sample size. We also added more information by performing a mediation analysis that supports our hypothesis that mitochondrial damage may be a causal intermediate in biological mechanisms linking air pollution exposure to birth outcomes, which may provide some mechanistic clues to the adverse effects of early exposure to air pollution observed in humans.

In conclusion, we have shown that prenatal NO_2 exposure was inversely associated with both placental mtDNA content and birth weight. Considering the high levels of NO_2 in urban areas, which are increasing worldwide, this study indicates the relevance of further exploring this biological pathway linking early air pollution exposure and complications at birth.

Acknowledgments and funding

We thank all the participants and collaborators in the INMA and ENVIR*ON*AGE cohorts. The research leading to these results was funded by the Spanish Ministry of Health (FIS-PI11/00610), by grants from UE (FP7-ENV-2011 cod 282957 and HEALTH.2010.2.4.5-1), Instituto de Salud Carlos III (Red INMA G03/176, CB06/02/0041, FIS-FEDER 03/1615, 04/1509, 04/1112, 04/1931, 05/1079, 05/1052, 06/1213, 07/0314, 09/02647, 11/01007, 11/02591, CP11/00178, FIS-PI06/0867, and FIS-PS09/00090), Conselleria de Sanitat Generalitat Valenciana, Spanish Ministry of Health (FIS-PI041436, FIS-PI081151, FIS-PI042018, FIS-PI09/02311), Generalitat de Catalunya-CIRIT 1999SGR 00241, Obre Social Cajastur, Universidad de Oviedo, Department of Health of the Basque Government (2005111093 and 2009111069), the Provincial Government of Gipuzkoa (DFG06/004 and DFG08/001).The ENVIRONAGE cohort is supported from the EU Program “Ideas” (ERC-2012-StG 310898) and the Flemish Fund for Scientific Research (FWO 1516112N and G073315N).

Supplementary material

Table S1. Data from each campaign and predictor variables of air pollution in each cohort

Cohort	No. Passive samplers	Campaign dates	Predictor variables	R^2 model	Number of monitoring stations
INMA-Asturias	67	June 2005 November 2005	Altitude Distance to nearest road[a] Agricultural or forest land cover (300m-buffer)	0.52	4
INMA-Gipuzkoa	85	February 2007 June 2005	Altitude (3 cat) Valley factor Distance to nearest road[a] (MDI[b] > 20000) Urban land cover (100m-buffer) Industrial land cover (300m-buffer)	0.51	3
INMA-Sabadell	57	April 2005 June 2005 October 2005 March 2006	Altitude Urban or industrial land cover (500m-buffer) Road type (minor, major, secondary road)	0.75	1
INMA-Valencia	93	April 2004 June 2004 November 2004 February 2005	Kriging[c] Industrial or urban land cover (500m-buffer) Distance to nearest major road[a] (MDI[b] > 10000)	0.73	7
ENVIRONAGE	N/A	N/A	Kriging[d] Land use[d] Road emission and locations[e] Point sources, characteristics and locations[e]	0.82	64

[a]Distance to the nearest major road (in logarithms)

[b]MDI: Mean daily traffic count

[c]Mean of estimated NO_2 from kriging among campains

[d]Predictor variables of the land-use regression model for the background (RIO)

[e]Predictor variables of the emission for the bi-gaussian plume model

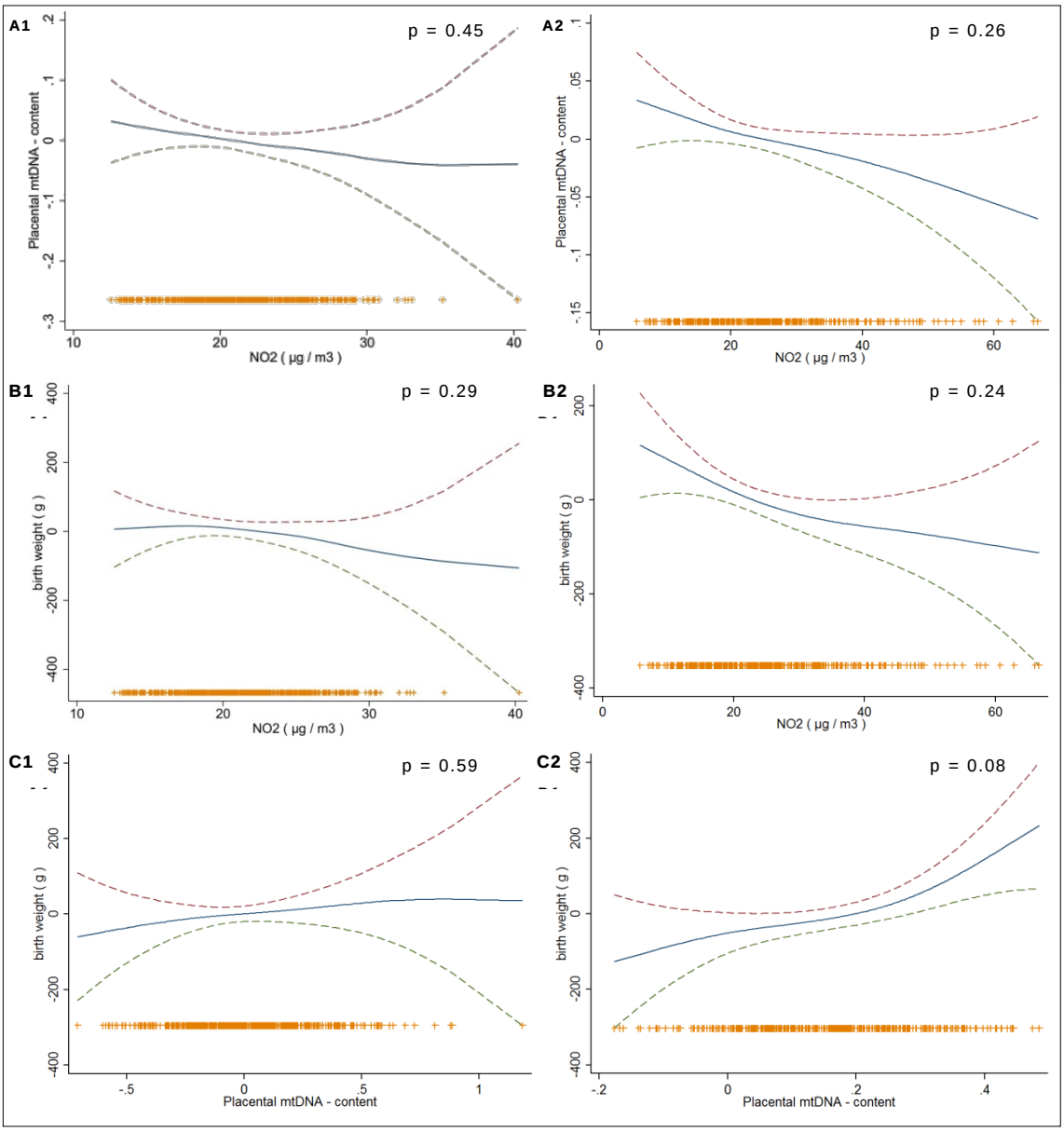

Figure S1. GAM models that show the relation between (A) NO_2 exposure (µg/m3) during the entire pregnancy and mtDNA content, (B) NO_2 exposure (µg/m3) during the entire pregnancy and birth weight (g), and (C) mtDNA content and birth weight (g). This model provides p-values for the null hypothesis of no linearity. 1: ENVIRONAGE, 2: INMA

Table S2. Percent change in placental mtDNA content in association with prenatal NO2 exposure in the four different INMA subcohorts.

Pregnancy period	Change in placental mtDNA content (%)	95% CI	P-value
Asturias (n=37)			
Trimester 1	-13.6	-31.4, 8.8	0.20
Trimester 2	-12.8	-26.0, 2.9	0.10
Trimester 3	-16.8	-30.8,-0.07	0.05
Entire pregnancy	-15.1	-29.9, 2.9	0.09
Gipuzkoa (n=156)			
Trimester 1	-6.3	-12.6, 0.5	0.07
Trimester 2	-6.5	-13.0, 0.4	0.06
Trimester 3	-5.4	-12.3, 2.1	0.15
Entire pregnancy	-4.6	-11.8, 3.2	0.23
Sabadell (n=120)			
Trimester 1	-2.6	-7.3, 2.3	0.29
Trimester 2	-3.4	-8.3, 1.8	0.16
Trimester 3	-3.4	-8.3, 1.8	0.19
Entire pregnancy	-4.1	-9.4, 1.6	0.15
Valencia (n=63)			
Trimester 1	-3.1	-8.5, 2.6	0.27
Trimester 2	-1.6	-8.1, 2.6	0.27
Trimester 3	-2.6	-8.3, 3.4	0.38
Entire pregnancy	-2.8	-8.9, 3.7	0.38

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, maternal smoking status, gestational age (linear and quadratic), pre-pregnancy BMI, parity, ethnicity, season of birth, and education

Table S3. Association between pregnancy average NO_2 exposure and birth weight in the four different INMA subcohorts.

INMA subcohort	N	Change in birth weight (g)	95% CI	*p*-value
Asturias	37	-155.6	-378.3, 67.1	0.16
Gipuzkoa	156	-75.6	-168.1, 13.1	0.08
Sabadell	120	-110.3	-178.3, -42.4	0.002
Valencia	63	-11.8	-20.1, 12.4	0.83

Effect size was estimated for each 10µg/m^3 increment in pregnancy average exposure to NO_2 at each mother's residence during the corresponding period.

Models were adjusted for newborn's sex, maternal age, maternal smoking status, gestational age (linear and quadratic), pre-pregnancy BMI, parity, ethnicity, season of birth, and education

Table S4. Mediation analysis of the estimated effects of prenatal NO_2 exposure (µg/m3) on birth weight through placental mtDNA content in the pooled sample and the boys of the pooled sample.

Exposure period	Proportion of mediation (%)	95% CI	*p*-value
Pooled data (n = 926)			
Trimester 1	1.4	-2.6, 5.5	0.54
Trimester 2	2.9	-2.6, 7.9	0.29
Trimester 3	3.2	-2.2, 8.1	0.24
Entire pregnancy	2.2	-2, 6.1	0.29
Boys of pooled data (n = 471)			
Trimester 1	1.5	-4.7, 7.0	0.62
Trimester 2	4.3	-0.2, 8.5	0.06
Trimester 3	6.4	2.4, 10.1	0.002
Entire pregnancy	2.8	-0.8, 6.2	0.12

Effect size was estimated for each 10µg/m^3 increment in pregnancy average exposure to NO_2 at each mother's residence during the corresponding period.

Models were adjusted for newborn's sex, maternal age, maternal smoking status, gestational age (linear and quadratic), pre-pregnancy BMI, parity, ethnicity, season of birth, and education

Table S5. Percent change in placental mtDNA content in association with prenatal NO2 exposure in INMA mothers that spent ≥ 15 hours a day at home.

Exposure period	N	Percent change (%)	95% CI	P-value
Trimester 1	214	-6.1	-9.5, -2.5	<0.01
Trimester 2	214	-7.5	-11.1, 3.8	<0.01
Trimester 3	214	-7.8	-11.4, -4.0	<0.01
Entire pregnancy	214	-8.9	-12.8, -4.8	<0.01

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, maternal smoking status, gestational age (linear and quadratic), pre-pregnancy BMI, parity, ethnicity, season of birth, and education;

Results followed the same direction in all 4 INMA subcohorts (data not shown);

INMA subcohort was included as random effect.

Table S6. Association between prenatal NO_2 and birth weight in INMA mothers that spent ≥ 15 hours a day at home.

Exposure period	N	Change in birth weight (g)	95% CI	P-value
Trimester 1	214	-64.9	-114.1, -15.6	0.01
Trimester 2	214	-65.9	-118.5, -13.3	0.01
Trimester 3	214	-75.1	-127.9, -22.2	< 0.01
Entire pregnancy	214	-83.7	-141.8, -25.6	< 0.01

Effect size was estimated for each 10$\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period.

Models were adjusted for newborn's sex, maternal age, maternal smoking status, gestational age (linear and quadratic), pre-pregnancy BMI, parity, ethnicity, season of birth, and education;

Results followed the same direction in all 4 INMA subcohorts (data not shown);

INMA subcohort was included as random effect.

CHAPTER 3

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

Diana B.P. Clemente[1,2,3,4], Maribel Casas[1,3,4], Bram G. Janssen[2], Aitana Lertxundi[5,6], Loreto Santa-Marina[4,6,7], Carmen Iñiguez[4,8,9], Sabrina Llop[4,9], Jordi Sunyer[1,3,4], Mònica Guxens[1,3,4,10], Tim S. Nawrot[2,11], Martine Vrijheid[1,2,4]

[1]ISGlobal, Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain
[2]Center for Environmental Sciences, Hasselt University, Diepenbeek, Belgium
[3]Universitat Pompeu Fabra, Barcelona, Spain
[4]CIBER de Epidemiologia y Salud Pública (CIBERESP), Institute of Health Carlos III, Madrid, Spain
[5]Universidad del País Vasco UPV-EUH, Spain
[6]Health Research Institute, Biodonostia, San Sebastián, Spain
[7]Public Health Division of Gipuzkoa, Basque Government, Spain
[8]Foundation for the Promotion of Health and Biomedical Research in the Valencian Region (FISABIO), Valencia, Spain
[9]Univerisity of Valencia, Valencia, Spain
[10]Department of Child and Adolescent Psychiatry/Psychology, Erasmus University Medical Centre – Sophia Children's Hospital, Rotterdam, The Netherlands
[11]Department of Public Health & Primary Care, Unit Environment & Health, Leuven University, Leuven, Belgium

Environmental Research 2017; 157:96–102

Abstract

Background The association between prenatal air pollution exposure and postnatal growth has hardly been explored. Mitochondrial DNA (mtDNA), as a marker of oxidative stress, and growth at birth can play an intermediate role in this association.

Objective In a subset of the Spanish birth cohort INMA we assessed first whether prenatal nitrogen dioxide (NO_2) exposure is associated with infant growth. Secondly, we evaluated whether growth at birth (length and weight) could play a mediating role in this association. Finally, the mediation role of placental mitochondrial DNA content in this association was assessed.

Methods In 336 INMA children, relative placental mtDNA content was measured. Land-use regression models were used to estimate prenatal NO_2 exposure. Infant growth (height and weight) was assessed at birth, at 6 months of age, and at 1 year of age. We used multiple linear regression models and performed mediation analyses. The proportion of mediation was calculated as the ratio of indirect effect to total effect.

Results Prenatal NO_2 exposure was inversely associated with all infant growth parameters. A 10 $\mu g/m^3$ increment in prenatal NO_2 exposure during trimester 1 of pregnancy was significantly inversely associated with height at 6 months of age (-6.6%; 95%CI: -11.4, -1.9) and weight at 1 year of age (-4.2%; 95%CI: -8.3, -0.1). 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. Furthermore, 5.5% (95%CI: 10.0, -0.2) of the association between trimester 1 NO_2 exposure and length at 6 months of age could be mediated by placental mtDNA content.

Conclusions Our results suggest that impaired fetal growth caused by prenatal air pollution exposure can lead to impaired infant growth during the first year of life. Furthermore, molecular adaptations in placental mtDNA are associated with postnatal consequences of air pollution induced alterations in growth.

Introduction

In the last decade, numerous studies have reported an association between prenatal ambient air pollution exposure and adverse birth outcomes. Prenatal nitrogen dioxide (NO_2) has been associated with low birth weight, intra-uterine growth retardation, and preterm birth, even at low levels of air pollution [29, 30]. The fetus may be particularly susceptible to air pollution exposure, because of its physiologic immaturity and its higher rates of cell proliferation [124]. The impact of NO_2 exposure on the fetus is important for public health because fetal growth, and birth size and weight of newborns are important predictors of the future health status during childhood and adulthood [1, 125]. Infant growth is believed to be a continuation of *in utero* growth and is influenced predominantly by factors determining intra-uterine growth and nutrition [126]; consequently, exposure to NO_2 during pregnancy could also affect infant growth. Nonetheless, little is known about how these intra-uterine effects may translate into variations in growth patterns of children after birth. Additionally, infant growth can be influenced by both genetic and environmental factors [127]. Adverse infant growth may be an important determinant of obesity and related health problems later in life [128, 129].

The placenta is a metabolically active organ that connects and separates two genetically distinct individuals: the mother and the fetus. It plays an essential role in nutrient transfer, growth and organ development. The placenta is a unique vascular organ that requires a constant source of energy. This energy provision is regulated by mitochondrial function of placental cells [110]. Mitochondria, the energy producers of the cells, are the major intracellular sources of reactive oxygen species (ROS), which are generated under normal conditions as by-product of oxidative phosphorylation. Mitochondria are the primary targets of oxidative stress because mitochondrial DNA (mtDNA) lacks protective strategies associated with nuclear DNA. Consequently, mitochondria are uniquely sensitive to environmental toxicants [81]. Furthermore, mtDNA content is correlated with the size and number of mitochondria, which have been shown to change under different energy demands, as well as different environmental conditions [77]. NO_2 has a strong oxidation capacity by generating ROS and reactive nitrogen species (RNS). Some studies have implicated that

mitochondrial function can be negatively affected by environmental toxicants stimulus, such as NO_2, $PM_{2.5}$, and black carbon [130-133]. Fetus adapt their mitochondrial structure and metabolism when the supply of nutrients is limited [112]. Changes in placental mtDNA content may represent a biological effect along the path linking air pollution to effects on the infant.

Recently, it was shown that placental mtDNA content was influenced by prenatal particulate matter <10µm (PM_{10}) and nitrogen dioxide (NO_2) exposure [113, 134]. Furthermore, in our previous study we showed that placental mtDNA content was significantly associated with birth weight and that it could be one of the mediators of the inverse association between prenatal NO_2 exposure and birth weight [134]. These findings raise the question of whether prenatal air pollution exposure may also result in subsequent changes in infant growth and whether placental mtDNA alterations can be linked to these outcomes in later life.

In the current study we therefore evaluated firstly whether prenatal NO_2 exposure is associated with infant growth (height and weight) at 6 months and 1 year of age. Secondly, we evaluated whether growth deficits at birth (length and weight) play a mediating role in this association. Finally, the mediating role of placental mtDNA content in the association between prenatal air pollution exposure and infant growth was assessed.

Methods

Study design and population

INMA (INfancia y Medio Ambiente; Environment and Childhood) is a birth cohort study that recruited pregnant women in seven regions, following a common protocol [135]. In this study we used participants with singleton live-born infants from three INMA regions (Valencia, Sabadell and Gipuzkoa). Pregnant women were enrolled between 2004 and 2008 during the first trimester of pregnancy at primary health care centers or public hospitals if they fulfilled the inclusion criteria: singleton pregnancy, intention to deliver at the reference hospital, ≥ 16 years of age, no problems of communication, and no assisted conception. Of all eligible women, 57% agreed to participate. Study approval was obtained from

the ethics committees of each participating center and informed consents were obtained from the mothers.

In INMA, placentas were randomly collected in approximately one of four deliveries (n = 502). The present analysis included 336 randomly selected mother-newborn pairs from whom placentas were collected and placental mtDNA content measured. The main characteristics of our study population including maternal age, smoking during pregnancy, maternal education, parity, gestational age, ethnicity, and prenatal NO_2 exposure are in line with the INMA participants that provided placental samples, but were not included in this study (Supplemental Material, Table S1 and Table S2). Therefore our population of mother-newborn pairs is representative for those who were not included in the analysis.

Ambient air pollution assessment

Ambient concentrations of nitrogen dioxide (NO_2) were measured with the aid of passive samplers (Radiello, Fundazione Salvatore Maugeri, Padua, Italy) installed in several sampling campaigns each lasting seven days and distributed across the study areas according to geographic criteria, taking into account the expected pollution gradients and the distribution of the residences of the participating women.

The methodology has been described in detail elsewhere [116, 117]. Briefly, area-specific land use regression (LUR) models were used to predict NO_2 levels at women's residential addresses, using the average of the NO_2 levels registered across campaigns to represent an annual mean level, together with land use (agricultural, industrial or urban), traffic-related variables, and altitude. Residential NO_2 estimations from LUR were then adjusted to time of pregnancy for each woman, using daily records from the monitoring network stations covering the study area. This model also took into account residential changes if women lived at least 2 months of pregnancy in the new residence. The validation statistics gave a spatial explained variance (R^2) for annual mean NO_2 from 0.51 to 0.75 in the three INMA regions [116].

In order to explore potentially critical exposures during pregnancy, individual NO_2 concentrations were calculated for different periods of pregnancy: trimester 1 (1-13 weeks), trimester 2 (14-28 weeks), trimester 3 (29 weeks to delivery), and for the entire pregnancy.

Placental mtDNA content

As previously described [134], placentas were entirely frozen after delivery at -20°C and afterwards at -86°C. Placentas were thawed minimally to obtain tissue biopsies for DNA extractions. To minimize the impact of within-placental variability, biopsies were all taken 1-1.5 cm below the chorio amniotic membrane at a fixed location and preserved at -80°C [113]. Briefly, DNA was extracted from placental tissue cells and quantified. MtDNA content was measured in placental tissue cells by determining the ratio of two mitochondrial gene copy numbers (mitochondrial encoded NADH dehydrogenase subunit 1 (*MT-ND1*) and mitochondrial forward primer for nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*)) to two single-copy nuclear control genes (acidic ribosomal phosphoprotein P0 (*RPLP0*), and beta-actin (*ACTB*)) using the 7900HT Fast Real-Time PCR System (Life Technologies, Foster City, CA, United States) [113]. Samples were run randomly in triplicate and each plate included six inter-run calibrators to account for inter-run variability. qBase software (Biogazelle, Zwijnaarde, Belgium) automatically averaged triplicate measurements that pass quality control and normalizes the data to nuclear reference genes while correcting for run-to-run differences [136].

Infant growth

Birth weight was recorded by trainee midwifes at delivery whereas birth length was measured by a nurse when the neonate arrived at the hospital ward within the first 12 hours of life.

Repeated height and weight measures from birth to 6 months of age were extracted from medical records. For infants without weight measures available within ± 14 days of their exact 6-month anniversary (n=17, 5.1% of the main analysis sample), we used the 2nd-order Reed sex-specific early infancy growth models to predict the weight of children as described previously [137]. Child height

and weight were measured at 1 year of age using standard protocols, with light clothing and without shoes. Age- and sex-specific z-scores for height and weight at birth, at 6 months and 1 year of age were calculated using the World Health Organization (WHO) referent [138]. The change in length and height z-score was calculated as the length/height z-score at follow up (6 months and 1 year of age) minus the length/weight z-score at birth divided by the timespan between birth and follow-up.

Covariates

Information on maternal age, ethnicity, education, smoking status, place of residence, pre-pregnancy BMI, and parity was obtained by self-reported questionnaires administrated by trained interviewers at 1st and 3rd trimester of pregnancy. Child sex and date of birth was obtained from clinical records.

Statistical analysis

Continuous data were checked for normality using the Shapiro-Wilk test statistic. Continuous data were presented as mean ± SD and categorical data as frequencies and percentages. Average placental mtDNA was log10-transformed to improve the normality of the distributions and described by geometric mean and 25th-75th percentile. Collinearity was assessed among the different exposure windows by calculating variance inflation factors (VIF). Multiple linear regression models were used to assess the association between (i) prenatal NO_2 exposure and infant growth (height and weight at 6 months and at 1 year of age) and between (ii) placental mtDNA content and infant growth.

Covariates used in the models were chosen *a priori*, including newborn's sex (male, female), gestational age (linear and quadratic term), maternal age (years), maternal pre-pregnancy BMI (kg/m^2), ethnicity (European, non-European), maternal education (primary, secondary, university), smoking during pregnancy (never, quit smoking before week 12, during entire pregnancy), parity (nulliparous, multiparous), season of birth (January-March, April-June, July-September, October-December), and region (Sabadell, Valencia, Gipuzkoa).

We presented adjusted models because they yielded similar results than the unadjusted ones.

Prior to the mediation analysis we explored if placental mtDNA content, birth weight and birth length were effect modifiers of the association between prenatal NO_2 exposure and height/weight at 6 months and 1 year of age. This was done by adding an interactionterm combining the NO_2 exposure with placental mtDNA content or interactionterms combining the NO_2 exposure with birth weight/length. Several mediation analyses were performed. Firstly, we investigated if birth length mediated the association between prenatal NO_2 exposure and length in the infants at 6 months and 1 year of age. Secondly, we assessed whether birth weight mediated the association between prenatal NO_2 exposure and weight in the infants at 6 months and 1 year of age. Thirdly, we investigated whether placental mtDNA content was a mediator of the association between prenatal NO_2 exposure and the different infant growth characteristics (height and weight at 6 months and 1 year of age). We only performed the mediation analysis when there was a significant association between the outcome and the exposure, a significant association between the exposure and the mediator, and a significant association between the outcome and the mediator. To perform these mediation analyses we used the SAS macro developed by Valeri and VanderWeele [121]. In this macro, the direct effect (DE), indirect effect (IE) and total effect (TE) were determined. The DE represents the effect of exposure on the outcome after controlling for the mediator whereas the IE is the effect of exposure operating through the mediator. The proportion of mediation was calculated as the ratio of IE to TE. For example, if the proportion of mediation is 0.32 this means that 32% of the total effect can be explained by the mediating variable in question.

Results

Characteristics and exposure levels of the study population

Table 1 summarizes the characteristics of the 336 mother-newborn pairs. Briefly, mean maternal age was 32.2 years. Pre-gestational BMI of the participating mothers averaged 23.5 kg/m² and 44.6% of the mothers never

smoked cigarettes. The newborns, 168 of which were girls (50.0%), had a mean gestational age of 39.9 weeks. More than 90% of the newborns were European.

Table 1. Study population characteristics (n=336)

Characteristics	Mean ± SD range or number (%)
Maternal	
Age, years	32.2 ± 3.9
Smoking	
Never	150 (44.6)
Quit smoking before week 12	128 (38.1)
During entire pregnancy	58 (17.3)
Education	
Primary school or none	68 (20.2)
Secondary school	154 (45.8)
University	114 (33.9)
Parity	
1	184 (54.8)
2	128 (38.1)
≥3	24 (7.1)
Pre-pregnancy BMI, kg/m^2	23.5 ± 4.3
Region	
Gipuzkoa	154 (45.8)
Sabadell	121 (36.0)
Valencia	61 (18.2)
Newborn	
Gestational age, weeks	39.9 ± 1.4
Sex	
Male	168 (50.0)
Female	168 (50.0)
Ethnicity	
European	306 (91.1)
Non-European	30 (8.9)
Season at birth	
January-March	76 (22.6)
April-June	89 (26.5)
July-September	88 (26.2)
October-December	83 (24.7)
Birth weight, g	3,284 ± 429
Weight at age 6 months, kg	7.7 ± 0.8
Weight at age 1 year, kg	9.8 ± 1.1
Birth length, cm	49.4 ± 2.1
Height at age 6 months, cm	67.2 ± 2.4
Height at age 1 year, cm	75.4 ± 2.8
Placental mtDNA content	1.5 (1.2-1.8)

Continuous covariates expressed by mean and standard deviation (SD) (normally distributed) or geometric mean and 25– 75th percentile (not normally distributed); categorical covariates described by numbers and frequencies (%).

Table 2 displays the daily outdoor NO_2 exposure levels averaged for the different exposure periods. Average (25th-75th percentile) period-specific NO_2 exposure was 27.0 (16.8-34.7) µg/m³ for trimester 1, 26.0 (16.7-32.6) µg/m³ for

trimester 2, 26.4 (17.0-33.4) µg/m³ for trimester 3, and 26.2 (17.4-33.3) µg/m³ for the entire pregnancy.

Table 2. Descriptive statistics of prenatal NO2 exposure (µg/m³) in the INMA study (N=336)

NO_2 exposure (µg/m³)	Mean ± SD	P5	P25	P50	P75	P95	Correlation[a] T1	T2	T3	EP
Trimester 1 (T1)	27.0 ± 13.0	5.6	16.8	24.8	34.7	74.2	1			
Trimester 2 (T2)	26.0 ± 11.9	5.7	16.7	24.7	32.6	74.7	0.85*	1		
Trimester 3 (T3)	26.4 ± 12.5	5.7	17.0	24.0	33.4	74.4	0.78*	0.85*	1	
Entire pregnancy (EP)	26.2 ± 11.6	5.7	17.4	24.6	33.3	66.7	0.91*	0.92*	0.94*	1

[a]Spearman correlation coefficients between different exposure periods

*P-value <0.0001

Association between prenatal NO_2 exposure and infant growth

Table 3 displays the percent change in z-scores for height and weight at 6 months and at 1 year of age for every 10 µg/m³ increment in NO_2 exposure during the different exposure windows of pregnancy. A 10 µg/m³ increment in prenatal NO_2 exposure was inversely and significantly associated with height at 6 months, especially during trimester 1 (-6.64%; 95%CI: -11.38, -1.90) and trimester 2 (-5.56%; 95%CI: -10.86, -0.26). Furthermore, each 10µg/m³ increment in NO_2 levels during trimester 1 of pregnancy was inversely and significantly associated with weight at 1 year (-4.21%; 95%CI: -8.34, -0.09). Prenatal NO_2 exposure was negatively but not significantly associated with weight at 6 months, and height at 1 year of age. VIF values in our collinearity diagnostics ranged from 4.74 to 7.39 for NO_2, indicating elevated levels of collinearity in the multi trimester models. These models show results in the same direction, but the confidence intervals are much wider (see Supplemental Materials, Table S3). The multi collinearity between the different exposure windows probably contribute to these wider confidence intervals that we observe.

Furthermore, prenatal NO_2 exposure was not associated with a change in weight z-scores between birth and 6 months/1 year of age and between 6 months and

1 year of age (see Supplemental Materials, Table S4). Only first trimester exposure to NO_2 was significantly positively associated with a change in length z-scores between 6 months and 1 year of age (0.8%; 95%CI: 0.17, 1.43). Prenatal NO_2 exposure was not significantly associated with length z-scores between birth and 6/12 months of age (see Supplemental Materials, Table S4).

Table 3. Association between maternal NO_2 exposure in different exposure periods of pregnancy and infant growth in the INMA study

	N	Change (%)	95% CI	P-value
zHeight at 6 months				
NO_2 Trimester 1	286	-6.64	-11.38, -1.90	<0.01
NO_2 Trimester 2	286	-5.56	-10.86, -0.26	0.04
NO_2 Trimester 3	286	-2.22	-7.13, 2.69	0.37
NO_2 Entire pregnancy	286	-5.20	-10.8, 0.41	0.07
zWeight at 6 months				
NO_2 Trimester 1	289	-3.32	-7.32, 0.67	0.10
NO_2 Trimester 2	289	-3.26	-7.6, 1.07	0.14
NO_2 Trimester 3	289	-2.16	-6.23, 1.92	0.30
NO_2 Entire pregnancy	289	-2.94	-7.62, 1.75	0.22
zHeight at 1 year				
NO_2 Trimester 1	286	-3.70	-9.58, 2.19	0.22
NO_2 Trimester 2	286	-3.53	-9.86, 2.54	0.24
NO_2 Trimester 3	286	-3.71	-9.96, 2.54	0.24
NO_2 Entire pregnancy	286	-4.51	-11.42, 5.97	0.20
zWeight at 1 year				
NO_2 Trimester 1	289	-4.21	-8.34, -0.09	0.04
NO_2 Trimester 2	289	-3.45	-7.99, 1.08	0.13
NO_2 Trimester 3	289	-2.47	-6.71, 1.77	0.25
NO_2 Entire pregnancy	289	-3.97	-8.84, 0.89	0.11

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education

Association between placental mtDNA content and infant growth

Table 4 shows that placental mtDNA content was significantly and positively associated with length at birth (0.29g; 95%CI: 0.04, 0.55). Furthermore, placental mtDNA content was significantly and positively associated with height at 6 months (5.90%; 95%CI: 0.60, 13.24) (Table 4). Placental mtDNA content was positively but not significantly associated with weight at 6 months, and with weight and height at 1 year of age. Furthermore, placental mtDNA content was not associated with a change in weight/height z-scores between birth and 6

months/1 year of age and between 6 months and 1 year of age (Data not shown).

Table 4. Association between placental mtDNA content and infant growth outcomes in INMA

Infant growth	N	Change	95% CI	P-value
Length at birth, cm	336	0.29	0.04, 0.55	0.02
Weight at birth, g[a]	336	73.8	18.60, 127.21	<0.01
zHeight at 6 months, %	286	5.90	0.60, 13.24	0.03
zWeight at 6 months, %	289	3.62	-1.34, 6.95	0.15
zHeight at 1 year, %	286	4.60	-1.50, 9.19	0.19
zWeight at 1 year, %	289	3.10	-1.25, 7.39	0.13

Effect size was estimated for each IQR (0.18) increase in placental mtDNA content;
Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education;
[a]These results were presented in a previous paper based on the same cohorts [134]

Mediation analyses

The added interactionterm combining the NO_2 exposure with placental mtDNA content and the interactionterms combining the NO_2 exposure with birth weight/length in the association between prenatal NO_2 exposure and the height/weight at 6 months and 1 year of age was not significant (data not shown). Meaning that neither placental mtDNA content nor birth weight/length were effect modifiers of the association between prenatal NO_2 exposure and infant growth.

We tested whether birth outcomes (birth length and weight) and mtDNA content could be mediators of the association between prenatal NO_2 exposure and infant growth. Prenatal NO_2 exposure during all the three trimesters of pregnancy and during the entire pregnancy was associated with birth weight, birth length, and mtDNA content (previous study [134] except for birth length and Supplemental Material, Table S5). Mediation analysis showed that birth length mediated 31.7% (DE = -4.78%, p = 0.02; IE = -2.39, p = 0.02) and placental mtDNA content 5.5% (DE = 5.6%, p = 0.02; IE = -0.32, p = 0.04) of the inverse association between prenatal NO_2 exposure during trimester 1 and infant height at age 6 months (Figure 1A).

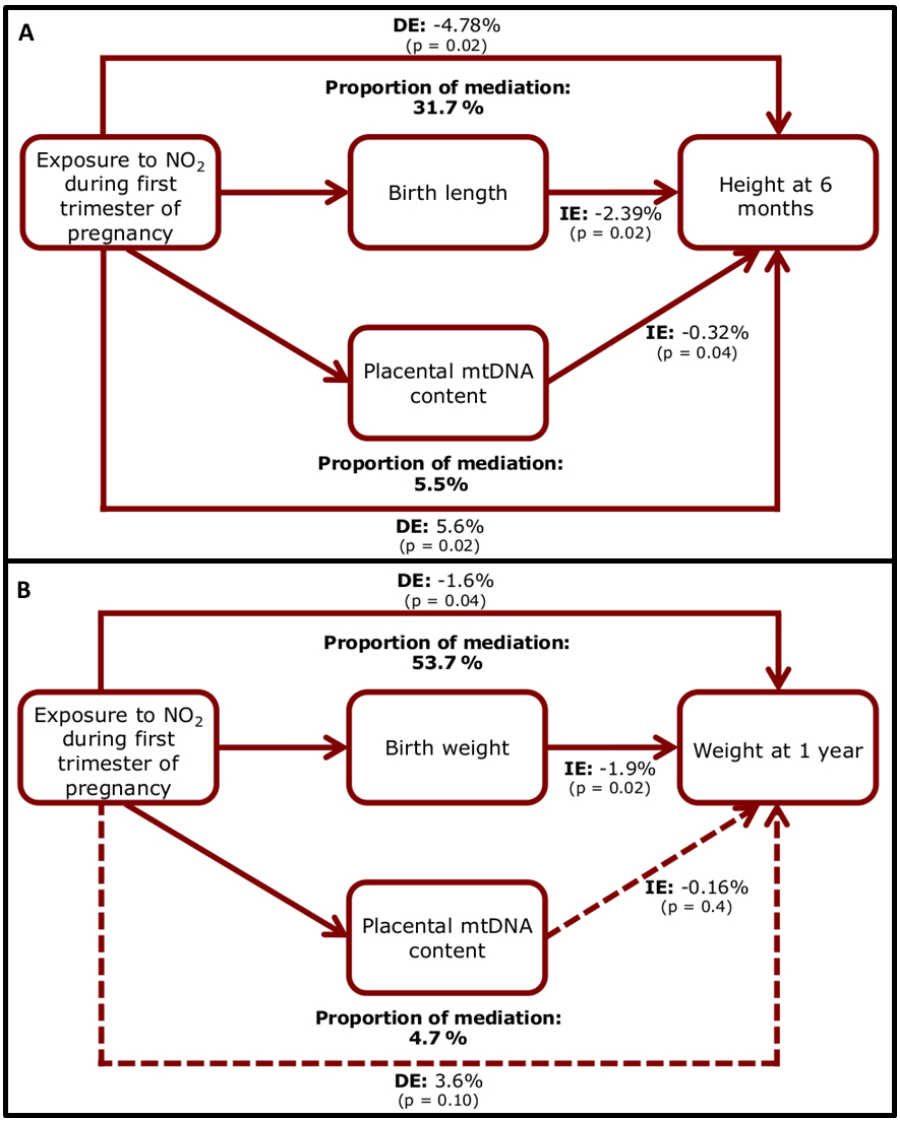

Figure 1. Mediation analyses. This figure shows the estimated proportion of the association between a 10 µg/m³ increment in NO_2 exposure during the first trimester of pregnancy and height at 6 months of age (A), and weight at 1 year of age, mediated through placental mtDNA content. Furthermore, it also includes mediation analysis showing the estimated proportion of the association between NO_2 exposure during the first trimester of pregnancy and both height at 6 months, mediated through birth length (A), and weight at 1 year of age, mediated through birth weight (B). The figure displays the estimates of the indirect effects (IE), the estimates of the direct effect (DE), and the proportion of mediation (IE/DE+IE).

Discussion

The present study indicates that prenatal air pollution exposure during early pregnancy results in significant growth deficits in newborns, and shows that these deficits continue to be seen at 6 months and 1 year of age. Furthermore, this study shows that growth at birth could mediate the effect of prenatal air pollution exposure on postnatal growth. Additionally, we showed that the association between prenatal NO_2 exposure and length at 6 months of age could be mediated by placental mtDNA content.

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 [139]. The analysis of birth outcomes in our study documented significant inverse associations between prenatal exposure to NO_2 and length of the newborn. Additionally, in a previous study we showed that birth weight was significantly inversely associated with prenatal exposure to NO_2 [134]. Several studies have estimated the impact of air pollution on anthropometric parameters at birth such as length, and weight [139-143]. An earlier observation in INMA on the participants from the region of Valencia reported a significant decrease in birth length of 0.27 cm (95% CI: -0.51, -0.03) [144]. Our birth outcome results are also consistent with the previous reported findings based on the same regions [123].

Exposure to air pollution during pregnancy may have long-term implications: Impaired fetal growth is believed to negatively influence infant growth and is also a risk factor for a number of adult chronic diseases such as cardiovascular diseases, and diabetes [125]. The results of this study show that air pollution exposure during the beginning of pregnancy is significantly negatively associated with height at 6 months of age and weight at 1 year of age. Our observation that prenatal NO_2 exposure during pregnancy appears to affect early postnatal growth in a negative manner is also consistent with two studies on the effect of smoking during pregnancy. A study in Brazil demonstrated that children exposed to maternal smoking during pregnancy showed persistent lower height-for-age from birth to adolescence compared to non-exposed [145]. Another study in Turkey showed that infants of mothers that smoked during pregnancy had significant weight and length deficits at birth compared with nonsmokers' infants.

Moreover, those infants continued to show significant deficits in height and weight at 6 months of age [146]. There are few studies that focused on the association between prenatal ambient air pollution and infant growth. There is one study that showed that infants exposed to higher traffic-related pollution in early life may exhibit more rapid postnatal weight gain (0-6 months) in addition to reduced fetal growth.[147] Another study in South Korea reported that prenatal PM_{10} exposure significantly lowered children's weight at 1 year of age [44]. The results of this study are consistent with our results that showed that prenatal NO_2 exposure is significantly negatively associated with infant weight at 1 year of age. As already mentioned, infant growth is believed to be a continuation of *in utero* growth [126]; this present study showed a mediation effect of fetal growth on the association between prenatal NO_2 and infant growth. This indicates that infant growth can be influenced by factors determining intra-uterine growth and nutrition. Nonetheless, a study with long-term follow-up observations is required to enhance the understanding of the association between prenatal ambient air pollution exposure and child's growth and to determine if intra-uterine effects may translate into variations in growth patterns during childhood.

The biological mechanisms whereby air pollutants might cause adverse growth effects are still unclear. Hypothesis are that oxidative stress and inflammation are important mechanisms in which air pollutants could cause adverse health outcomes [148]. Mitochondria are uniquely sensitive to environmental toxicants that induce oxidative stress, such as air pollutants. MtDNA is particularly vulnerable to ROS-induced damage and has been described as a proxy of air pollution-induced damage [82, 149]. MtDNA has a high mutation rate [150] and mitochondria compensate for these mutations by altering their mtDNA content (each mitochondria carries 2-10 copies of mtDNA) [82, 151]. In a subsequent study combining the Belgian ENVIR*ON*AGE birth cohort with the INMA birth cohort, we demonstrated that mtDNA content was one of the potential mediators between the association of prenatal air pollution exposure and birth weight [134]. This current study adds information by showing that 5.5% of the association between NO_2 exposure during the first trimesters of pregnancy and length at 6 months of age could be mediated through placental mtDNA content. This may indicate that a decrease in mtDNA content in early life could lead to impaired growth trajectories up to six months of age, which might have health consequences in

later life. Furthermore, mitochondrial dysfunction can be caused by a change in mtDNA content and a decrease in mtDNA content has been related to the development of multiple forms of disease as type 2 diabetes [85, 152], breast cancer [153], and low birth weight [112, 134].

Cross-sectional human studies on the association between ambient air pollution exposure and mtDNA content are still limited with inconsistent results. PM air pollution exposure was associated with an increase [82], a decrease [154, 155], and no change in mtDNA content [152] in adults and elderly. Additionally, increased mtDNA content in adults has been associated with short-to moderate-term ambient black carbon (BC) levels [156] and benzene exposure [157]. Studies investigating the effect of ambient air pollution exposure during pregnancy on placental mtDNA content are limited to maternal tobacco smoke exposure [151, 158]. Although, recently a significant inverse association between prenatal $PM_{2.5}$ exposure and lower mtDNA content in cord blood was found [159]. The previously mentioned discrepancy in the mtDNA content results, can be explained by the very dynamic nature of mtDNA. MtDNA content most importantly depends on oxidative stress level, type of environmental factor, cell antioxidant capacity and dose of exposure, additionally, mtDNA content also fluctuates under the influence of age, ethnicity, and the tissue investigated [160, 161]. The current hypothesis of this discrepancy 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 [79]. 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 [162].

In the present study we have found that birth length mediates the association between prenatal NO_2 exposure and infant length at 6 months. Additionally, we also showed that mtDNA content mediates the association between prenatal NO_2 exposure and birth length (6.20%; 95%CI: 9.5, 0.4). Consequently, this can mean that the mediating role of placental mtDNA content could be partially independent by mediating the association between prenatal NO_2 exposure and infant length at 6 months of age and partially dependent through its mediating effect of prenatal NO2 exposure on birth length. Furthermore, no significant

association could be found between prenatal NO_2 exposure and length at 1 year of age. However, we found that prenatal NO_2 exposure was significantly positively associated with a change in length z-score between 6 months and 1 year of age. This could lead to the hypothesis that the child will undergo a height catch-up during this period which could explain the non-significant effect of prenatal NO_2 exposure on height at 1 year of age observed in this study. According to the literature, the period of life between 6 months and 2 years had been noted as a period of critical height development [163]. Height growth rather than fat storage may be stimulated preferentially during this period [164]. This could explain why prenatal NO_2 exposure was associated with length and not with weight at 6 months of age.

We acknowledge several limitations in the present study. Our placental mtDNA content associations should be interpreted cautiously within the context of cellular heterogeneity. All tissues are composed of multiple cell types and the mtDNA content in a tissue is an average of the mtDNA content in all existing cell types. The placenta is composed of a complex population of cells [mesenchymal cells, mesenchymal derived macrophages (Hofbauer cells), fibroblasts, smooth muscle cells, perivascular cells (pericytes), and endothelial cells] [165]. There is a possibility that placental mtDNA content data is confounded by variation in cell type distributions and may not reflect true mtDNA content differences, but only differences in cell type composition. To overcome this caveat, it is necessary to characterize and explore the effects of cellular heterogeneity in heterogeneous tissues such as the placenta [166]. In whole blood, it is possible to correct for differences in blood composition using algorithms that estimate cell type proportions [166, 167]. Currently, there is no algorithm available to estimate the cell type proportions in placental tissue. However, to minimize the impact of within placental variability, biopsies used for mtDNA content assays were all taken 1-1.5 cm below the chorio-amniotic membrane at a fixed location. Care was taken by visual examination and dissection to avoid the chorio-amniotic membrane contamination. Histological confirmation of cell type showed no difference in cell type composition between the fetal samples taken at four standardized sites across the middle region of the placenta (approximately 4 cm away from the umbilical cord), nor between the four placentas. Furthermore, although our results were consistent after multiple adjustments, we cannot exclude that our

findings were caused by some unknown factor that is associated with prenatal air pollution exposure, placental mtDNA content and infant growth. Thirdly, although we used a recently developed statistical mediation method [121], this method cannot prove the biological direction (causality); nevertheless, our formal mediation analysis is based on a predefined hypothesis and is in line with experimental evidence. Fourthly, as discussed previously, other studies showed an effect of other air pollutants (such as PM and BC) on mtDNA content. In INMA, NO_2 was the only ambient exposure that was available for all regions during pregnancy and we were not able to account for other exposures. Therefore, confounding due to co-pollutants (PM, BC, and others) may have introduced bias in the present study. Nonetheless, NO_2 is frequently used as a surrogate for traffic related air pollution because it is considered to be a good proxy of other pollutants originating from the same sources [168]. Finally, we need to consider that the prenatal NO_2 exposure assessment was limited to the residential address of the mothers and did not consider the individual's time based activity patterns; therefore, the measure of NO_2 exposure could be inaccurate for the mothers that stayed outside their living area for a longer time period. However, there was no significant difference in the associations when we restricted our analysis to mother who spend > 15 hr/day at home (data not shown).

In conclusion, this study suggests that prenatal air pollution exposure can lead to impaired infant growth that is determined by intra-uterine growth. Additionally, air pollution induced alterations in placental mtDNA, indicating a biological oxidative stress pathway involving the placenta, might have consequences to growth up to six months of age.

Acknowledgments and funding

ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya. The research leading to these results was funded by the Spanish Ministry of Health (FIS-PI11/00610).

Supplementary material

Table S1. Comparison of the characteristics of INMA participants that provided placenta samples included in this study (n = 336) with those that were not included in this study (n = 166).

	INMA participants that provided placenta samples included in this study (n = 336)	INMA participants that provided placenta samples, but were not included in this study (n = 166)
Characteristics	**Mean ± SD range percentage (%)**	**Mean ± SD range or percentage (%)**
Maternal		
Age, years	32.2 ± 3.9	32.1 ± 4.3
Smoking		
Never	44.6	46.1
Quit smoking before week 12	38.1	33.9
During entire pregnancy	17.3	20.0
Education		
Primary school or none	20.2	21.1
Secondary school	45.8	40.7
University	33.9	38.1
Parity		
1	54.8	54.5
2	38.1	37.2
≥3	7.1	8.3
Pre-pregnancy BMI, kg/m^2	23.5 ± 4.3	23.7 ± 4.2
Newborn		
Gestational age, weeks	39.9 ± 1.4	39.8 ± 1.3
Sex		
Male	50.0	56.2
Female	50.0	43.8
Ethnicity		
European	91.1	91.5
Non-European	8.9	8.5

Continuous covariates expressed by mean and standard deviation (SD) (normally distributed); categorical covariates described by numbers and frequencies (%).

Table S2. Comparison of the prenatal NO2 exposure (µg/m³) during the different exposure windows of pregnancy and the entire pregnancy of INMA participants that provided placenta samples included in this study (n = 336) with those that were not included in this study (n = 166).

	INMA participants that provided placenta samples included in this study (n = 336)						INMA participants that provided placenta samples, but were not included in this study (n = 166)						
NO_2 exposure ($\mu g/m^3$)	**Mean ± SD**	**P5**	**P25**	**P50**	**P75**	**P95**	**Mean ± SD**	**P5**	**P25**	**P50**	**P75**	**P95**	**P-value[a]**
Trimester 1	27.0 ± 13.0	5.6	16.8	24.8	34.7	74.2	24.6 ± 11.4	9.6	16.5	21.5	32.0	45.7	0.08
Trimester 2	26.0 ± 11.9	5.7	16.7	24.7	32.6	74.7	25.2 ± 9.4	11.1	17.7	25.9	30.2	40.2	0.46
Trimester 3	26.4 ± 12.5	5.7	17.0	24.0	33.4	74.4	25.7 ± 10.6	11.4	17.9	24.0	29.9	46.5	0.56
Entire pregnancy	26.2 ± 11.6	5.7	17.4	24.6	33.3	66.7	25.1 ± 9.4	11.1	18.5	24.4	29.7	41.9	0.31

[a]Differences between cohorts were assessed using independent t-tests.

Table S3. Association between maternal NO2 exposure in different exposure periods of pregnancy and infant growth in a multi trimester model

	N	Change (%)	95% CI	P-value
zHeight at 6 months				
NO_2 Trimester 1	286	-7.65	-15.62, 0.31	0.06
NO_2 Trimester 2	286	-2.48	-13.05, 8.1	0.64
NO_2 Trimester 3	286	-4.5	-12.03, 3.03	0.24
zWeight at 6 months				
NO_2 Trimester 1	289	-2.58	-9.19, 4.02	0.44
NO_2 Trimester 2	289	-1.48	-9.78, 6.83	0.73
NO_2 Trimester 3	289	0.54	-5.62, 6.70	0.86
zHeight at 1 year				
NO_2 Trimester 1	286	-0.28	-10.81, 10.25	0.96
NO_2 Trimester 2	286	-1.87	-14.23, 10.48	0.77
NO_2 Trimester 3	286	-1.09	-11.16, 8.98	0.83
zWeight at 1 year				
NO_2 Trimester 1	289	-3.97	-10.88, -2.94	0.26
NO_2 Trimester 2	289	-0.5	-9.19, 8.19	0.91
NO_2 Trimester 3	289	0.67	-5.78, 7.11	0.84

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, education, and NO_2 exposure during the other two trimesters.

Table S4. Association between prenatal NO2 in different exposure periods of pregnancy and change in weight/length Z-scores between birth and 6months/ 1 year of age and between 6 months and 1 year of age.

	N	Change (%)	95% CI	P-value
Change in weight z-scores between birth and 6 months of age				
NO_2 Trimester 1	286	0.38	-0.40, 1.59	0.34
NO_2 Trimester 2	286	0.47	-0.39, 1.33	0.28
NO_2 Trimester 3	286	0.24	-0.57, 1.04	0.56
NO_2 Entire pregnancy	286	0.51	-0.42, 1.43	0.28
Change in weight z-scores between birth and 1 year of age				
NO_2 Trimester 1	289	0.13	-0.28, 0.55	0.52
NO_2 Trimester 2	289	0.22	-0.23, 0.68	0.33
NO_2 Trimester 3	289	0.11	-0.31, 0.53	0.61
NO_2 Entire pregnancy	289	0.19	-0.30, -0.68	0.45
Change in weight z-scores between 6 months and 1 year of age				
NO_2 Trimester 1	281	-0.11	-0.45, 0.23	0.52
NO_2 Trimester 2	281	-0.02	-0.39, 0.36	0.92
NO_2 Trimester 3	281	-0.01	-0.37, 0.34	0.93
NO_2 Entire pregnancy	281	-0.13	-0.53, 0.28	0.54
Change in length z-scores between birth and 6 months of age				
NO_2 Trimester 1	286	-0.23	-1.16, 0.71	0.63
NO_2 Trimester 2	286	0.32	-0.73, 1.37	0.55
NO_2 Trimester 3	286	0.19	-0.77, 1.14	0.70
NO_2 Entire pregnancy	286	0.15	-0.95, 1.25	0.79
Change in length z-scores between birth and 1 year of age				
NO_2 Trimester 1	289	0.30	-0.25, 0.95	0.28
NO_2 Trimester 2	289	0.35	-0.25, 0.95	0.25
NO_2 Trimester 3	289	0.14	-0.46, 0.73	0.66
NO_2 Entire pregnancy	289	0.31	-0.35, 0.97	0.36
Change in length z-scores between 6 months and 1 year of age				
NO_2 Trimester 1	281	0.80	0.17, 1.43	0.01
NO_2 Trimester 2	281	0.45	-0.26, 1.16	0.21
NO_2 Trimester 3	281	0.11	-0.59, 0.8	0.76
NO_2 Entire pregnancy	281	0.36	-0.41, 1.13	0.36

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education

Table S5. Association between prenatal NO2 in different exposure periods of pregnancy and birth length, birth weight and placental mtDNA content

	Change	95% CI	P-value
Birth length, cm[a]			
NO_2 Trimester 1	-0.27	-0.46, -0.08	<0.01
NO_2 Trimester 2	-0.21	-0.40, -0.01	0.04
NO_2 Trimester 3	-0.21	-0.40, -0.01	0.03
NO_2 Entire pregnancy	-0.27	-0.49, -0.05	0.01
Birth weight, g			
NO_2 Trimester 1	-61.9	-102.2, -21.7	<0.01
NO_2 Trimester 2	-61.2	-103.1, -19.3	<0.01
NO_2 Trimester 3	-58.2	-100.2, -16.1	<0.01
NO_2 Entire pregnancy	-73.4	-120.4, -26.4	<0.01
Placental mtDNA content, %[a]			
NO_2 Trimester 1	-3.5	-6.5, -0.4	0.03
NO_2 Trimester 2	-3.9	-7.0, -0.7	0.02
NO_2 Trimester 3	-3.9	-7.1, -0.7	0.02
NO_2 Entire pregnancy	-4.5	-7.9, -0.9	0.02

Effect size was estimated for each 10 $\mu g/m^3$ increment in exposure to NO_2 at each mother's residence during the corresponding period;

Models were adjusted for newborn's sex, maternal age, smoking status, gestational age, gestational age^2, maternal pre-pregnancy BMI, parity, ethnicity, season, and education;

[a]These results were presented in a previous paper based on the same cohorts [134]

CHAPTER 4

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

Diana B.P. Clemente[1,2,3], Martine Vrijheid[1,3,4], Dries S. Martens[2], Mariona Bustamente[1,3,4], Leda Chatzi[5,6,7], Asta Danileviciute[8], Regina Grazuleviciene[8], Kristine B. Gutzkow[9], Lea Maitre[1,3,4], Rosie R.C. McEachan[10], Oliver Robinson[11], Per E. Schwarze[9], Ibon Tamayo [12], Marina Vafeiadi[6], John Wright[10], Rémy Slama[13], Mark Nieuwenhuijsen[1,3,4], Tim S. Nawrot[2,14]

[1]ISGlobal, Center for Research in Environmental Epidemiology (CREAL), Barcelona, Spain

[2]Center for Environmental Sciences, Hasselt University, Hasselt, Belgium

[3]Universitat Pompeu Fabra, Barcelona, Spain

[4]CIBER de Epidemiologia y Salud Pública (CIBERESP), Institute of Health Carlos III, Madrid, Spain

[5] Department of Preventive Medicine, University of Southern California, Los Angeles, CA, United States

[6]Department6Department of Social Medicine, University of Crete, Greece

[7]NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, Netherlands

[8]Department of Environmental Science, Vytauto Didziojo Universitetas, Kaunas, Lithuania

[9]Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway.

[10]Bradford Institute for Health Research, Bradford Royal Infirmary, Bradford, UK

[11]MRC-PHE Centre for Environment and Health, School of Public Health, Imperial College London, UK

[12]Department of Statistics, Faculty of Arts and Sciences, Harvard University, Cambridge, Massachussets, USA

[13]Inserm and University Grenoble-Alpes, U1209, IAB, Team of Environmental Epidemiology Applied to Reproduction and Respiratory Health, Grenoble, France

[14]Department of Public Health & Primary Care, Unit Environment & Health, Leuven University, Leuven, Belgium

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 [148].

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 [58]. 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 [68, 169, 170], type 2 diabetes [171] and increased mortality [172-174]. Furthermore, it is believed that the natural erosion of telomeres is accelerated through oxidative stress and inflammation [175].

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, 104]. The rate of telomere attrition is greatest in young children [176] and the telomere length decline then continues at a slower rate throughout adulthood [177] 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 [98], the Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) study in France [99], the INfancia y Medio Ambiente (INMA) cohort in Spain [97], the Kaunus cohort (KANC) in Lithuania [100], the Norwegian Mother and Child Cohort Study (MoBa) [101] and the RHEA Mother Child Cohort study in Crete, Greece [102]. 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 characterized for a broad suite of environmental exposures, to be clinically examined, and to have biological samples collected. A new follow-up visit was organized 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 [178]. Telomere and single copy-gene reaction mixture and PCR cycles used can be found in Martens et al [179]. 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 [180, 181]. In EDEN, dispersion models were used to assess the NO_2 exposure [182] and the ESCAPE European-wide LUR model was applied for $PM_{2.5}$, corrected for local background monitoring data [183]. 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 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	
No	1121 (83.3)
Yes	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 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 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).

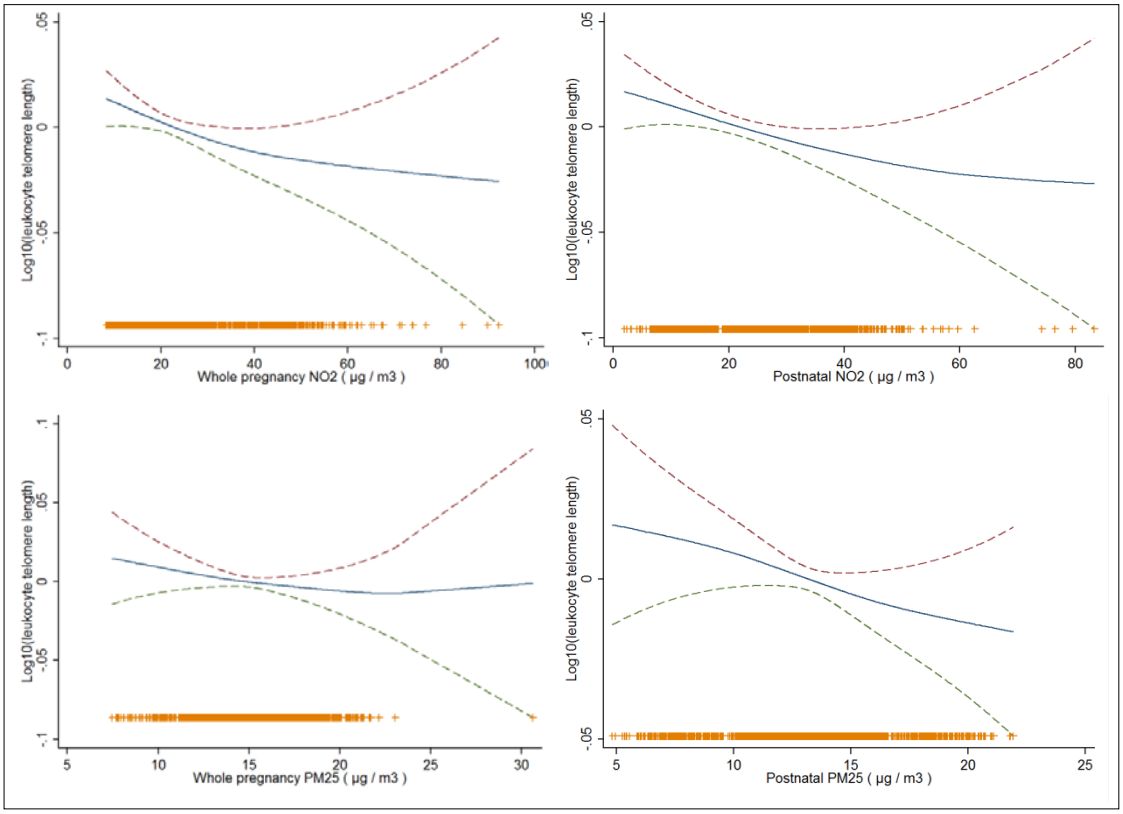

Figure 1. GAM models show the linear relation between (A) Prenatal NO_2 exposure (µg/m3) during the entire pregnancy and child leukocyte telomere length, (B) Postnatal NO_2 exposure (µg/m3) and child leukocyte telomere length, and (C) $PM_{2,5}$ exposure (µg/m3) during the entire pregnancy and child 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 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.

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).

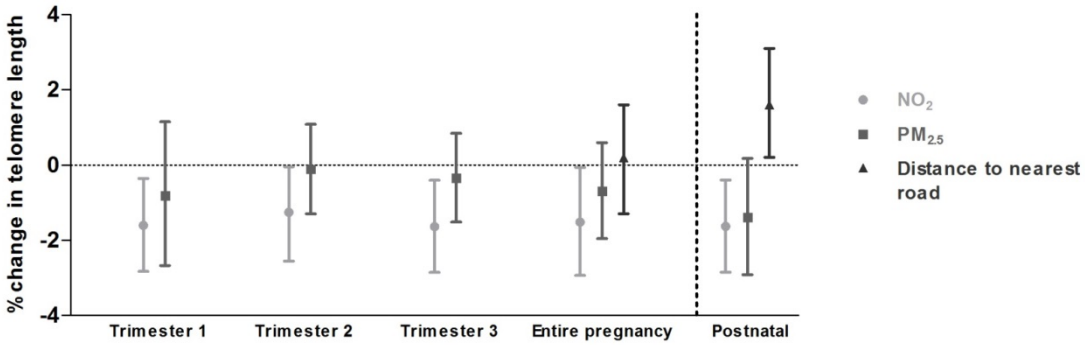

Figure 2. The association leukocyte telomere length between prenatal (trimester specific and whole pregnancy)/postnatal traffic-related air pollution/distance to nearest road and in 8-year old children; Effect size (% change with 95%CI) was estimated for each SD increment in exposure to NO_2, and $PM_{2.5}$ 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, 184, 185]. 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) [186]. A Canadian study [187] 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 $\mu g/m^3$ increment in $PM_{2.5}$ exposure [184]. Conversely, improvements in air pollution parallel increases in the US population life-expectancy which could not be attributed to demographic or social economical changes [188].

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 [189]. It is assumable that telomeres are a sensitive target for ROS-induced damage, as telomeres contain a high amount of ROS sensitive guanine bases[190]. ROS can induce DNA breakage, and single strand breaks in telomeric DNA is ineffectively repaired, leading to increased telomere shortening [191].

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 [192, 193]. 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 [192]. 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 $\mu g/m^3$ increase in residential $PM_{2.5}$ exposure, respectively [193]. 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 [155, 194-196], 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 [197]. 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 [198, 199] 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)[194]. 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 [195]. Furthermore, a study in the KORA F4 cohort found that telomere length was inversely associated with black carbon in men ($\beta = -0.28$; 95% CI = $-0.47, -0.1$)[200].

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 [201] and the annual telomere loss in adult leukocytes is between 32.2 and 45.5 bp [202]. 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 [203, 204]. 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 [205], 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.

Funding

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 308333 – the HELIX project. Tim S. Nawrot was funded by the EU Program "Ideas" (ERC-2012-StG 310898).

Aknowledgements

ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya. We are grateful to all the participating families in the six countries who took part in this study.

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
EDEN								
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

Diana B.P. Clemente[1,2,3], Lea Maitre[1,3,4], Mariona Bustamente[1,3,4], Leda Chatzi[5], Asta Danileviciute[6], Serena Fossati[1,3,4], Rosie R.C. McEachan[7], Helle Meltzer[8], Inga Petraviciene[6], Rémy Slama[9], Ibon Tamayo[10], Cathrine Thomsen[8], Marina Vafeiadi[5], John Wright[7], Tim Nawrot[2,11], Martine Vrijheid[1,3,4]

[1]ISGlobal, Institute for Global Health, Barcelona, Spain

[2]Center for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

[3]Universitat Pompeu Fabra, Barcelona, Spain

[4]CIBER Epidemiología y Salud Pública (CIBERESP), Spain.CIBER Epidemiología y Salud Pública (CIBERESP), Spain.

[5]Department of Social Medicine, University of Crete, Greece

[6]Department of Environmental Science, Vytauto Didziojo Universitetas, Kaunas, Lithuania

[7]Bradford Institute for Health Research, Bradford Royal Infirmary, Bradford, UK

[8]Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, NO-0403 Oslo, Norway.

[9]Inserm and University Grenoble-Alpes, U1209, IAB, Team of Environmental Epidemiology Applied to Reproduction and Respiratory Health, Grenoble, France

[10]Department of Statistics, Faculty of Arts and Sciences, Harvard University, Cambridge, Massachussets, USA

[11]Department of Public Health & Primary Care, Unit Environment & Health, Leuven University, Leuven, Belgium

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, shorter telomeres have been associated with obesity in adults but information is lacking in children. In this study, we evaluated the association between various maternal and child indices of obesity with telomere length in childhood.

Methods In 1,396 mother-child pairs of the multi-centre European birth cohort study HELIX, maternal pre-pregnancy body mass index (BMI) and 4 adiposity markers were assessed in children at age 8 years (6-11): BMI, fat mass from bioimpedence measurements, waist circumference, and skinfold thickness determined as the sum of subscapular and triceps skinfold thickness. Relative leukocyte telomere length (LTL) was measured by real time polymerase chain reaction (qPCR). Associations of telomere length with each adiposity marker (z-scores) were calculated using 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 LTL was 0.23% shorter (95% confidence interval, CI: 0.01, 0.46%). Each unit increase in child BMI z-score was associated with 1.21% (95% CI: 0.30, 2.11%) shorter LTL. Adding maternal pre-pregnancy BMI and child BMI in the same model did not substantially change our reported associations for child BMI but did alter the associations for maternal BMI. Inverse associations of borderline statistical non-significance were observed between child waist circumference and LTL (-0.96% per z-score unit; 95% CI: -2.06, 0.16%), and skinfold thickness and LTL (-0.10% per z-score unit; ; 95% CI: -0.23, 0.02%).

Conclusion Obesity markers in children or their mothers are associated with shorter telomere length in children around age 8. Furthermore, child BMI was more strongly associated with shorter telomere length than maternal pre-pregnancy BMI. Obesity may accelerate telomere shortening in children and thus may accelerate cellular aging.

Introduction

There is a major global obesity epidemic [206, 207]. Obesity is a risk factor for increased morbidity and mortality in adulthood. Obesity has been consistently associated with increased systemic inflammation and oxidative stress [208, 209], which are also causes of telomere shortening in cells [76, 210]. Telomeres are nucleoprotein structures containing tandem repeats of DNA (5'-TTAGGG-3'), situated at the termini of the chromosomes [211]. Telomeres function in maintaining the integrity of chromosomes and the stability of the genome, and to prevent end-to-end chromosomal fusions [212]. 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. Shorter telomeres have been associated with age-related diseases such as cardiovascular disease [68, 169, 170], type 2 diabetes [171] and increased mortality [172-174]. Telomere length variability and attrition rate has been explained by heritability and by different environmental determinants [155, 213-216]. In studies with adult subjects, obesity and other pro-inflammatory risk factors have been associated with shorter leukocyte telomere length [155, 216-219]. In children, however, case-control studies of telomere length and childhood obesity have produced conflicting results, with some showing that obesity is related to shorter telomeres but others finding no association [220-222].

Recent findings have shown that newborn telomere length may be influenced by several intrauterine effects, such as air pollution exposure [193, 223-227]. Additionally, Martens *et al* (2016) showed that pre-pregnancy BMI is associated with shorter newborn cord blood and placental telomeres [179].

The rate of telomere attrition is greatest in young children [176] and telomere length decline, then continues at a slower rate throughout adulthood [177]. 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 approximately 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 [103] 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 [98], the Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant (EDEN) study in France [99], the INfancia y Medio Ambiente (INMA) cohort in Spain [97], the Kaunus cohort (KANC) in Lithuania [100], the Norwegian Mother and Child Cohort Study (MoBa) [101] and the RHEA Mother Child Cohort study in Crete, Greece [228]. 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) sufficiently 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). Finally we focused on mother-child pairs with complete study-questionnaire and clinical examination data, and urine and blood samples (n = 1396).

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) [229] 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, 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

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 [178]. Telomere and single copy-gene reaction mixture and PCR cycles used can be found in Martens et al [179]. 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/m^2). In adults, 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. In children from 5-19 years, a BMI-for-age value within the range of -2SD and +1SD is categorized as normal, within +1SD and +2SD as overweight and ≥ 2SD as obese [50]. 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 [50]. 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 was measured to the nearest 0.1 mm at four points (triceps, subscapular, suprailiac, and quadriceps) following standardized procedures. The sum of skinfold thickness of two 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 bioimpedence 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. Fat mass was standardized using internal z-score for age and sex.

Statistical analysis

Continuous data were checked for normality. Average relative telomere lengths showed a skewed distribution and were $\log_{10}$ transformed to improve normality. The obesity parameters were treated as both continuous (z-score unit) and categorical variables in models evaluating 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. Multivariable linear mixed models with a random cohort effect were used to address the different associations. All analyses were adjusted for *a priori*

chosen covariates including maternal education, maternal age at birth, child's age in days, sex, batch (2 categories), cohort, child's ethnicity, birth weight, maternal smoking during pregnancy, and blood cell type proportions. 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).

In a first sensitivity analysis we used the complete data instead of the imputed data for our initial analyses. Additionally we used a model that included both maternal pre-pregnancy BMI and child BMI as explanatory variables. Further, we stratified our analysis of 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 = 1,396). The children had a mean (SD) age of 8 (1.5) years, 753 (53.9%) were boys, they were mainly from white European origin (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. A total of 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 of children of white European origin (43%). The children in BIB, RHEA and KANC were the youngest (approximately 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 pre-pregnancy BMI was significantly correlated with the different child obesity parameters. Child obesity parameters were highly associated with one another.

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). Figure 1 shows the linear relationship of the association between child telomere length and maternal pre-pregnancy BMI and child BMI z-score. 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%). Additionally, analyses using the maternal pre-pregnancy BMI as categorical variables showed that compared to children with mothers with a normal pre-pregnancy weight, telomere length was not significantly shorter in children of mothers with pre-pregnancy overweight and obesity (Table 4).

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.

Child's telomere length was also inversely associated with obesity parameters measured in the children, including BMI, waist circumference, skinfold 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 associations of borderline statistical significance 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 clearly associated with telomere length (-0.65% per unit increase; 95% CI: -1.76, 0.48).

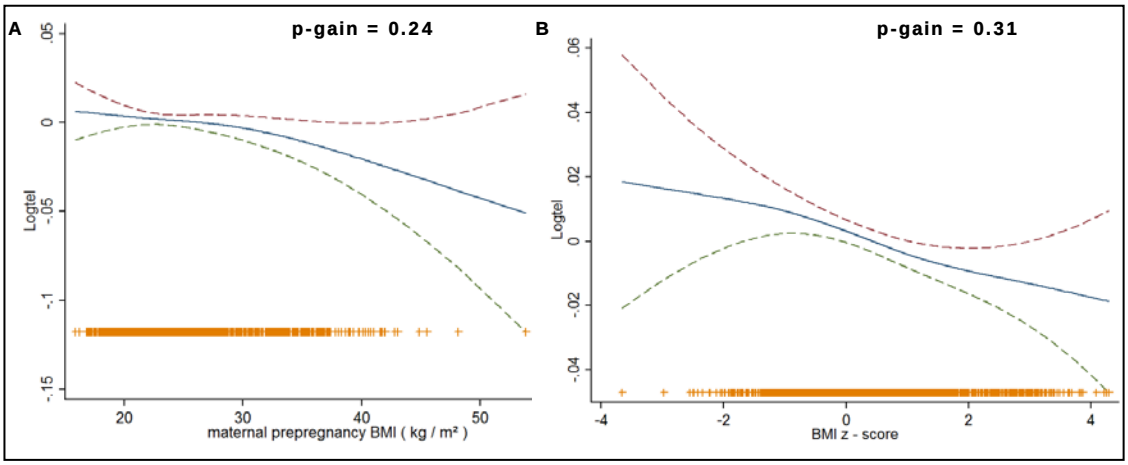

Figure 1. GAM models that show the relation between child telomere length and (A) maternal pre-pregnancy BMI and (B) child BMI

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.

Additionally, analyses using the obesity parameters as categorical variables showed that compared to children with a normal weight, telomere length was shorter in overweight and obese children (Table 4).

In a sensitivity analysis using the complete data we obtained exactly the same results as the analyses with the imputed data (data not shown). Additionally, we have included an analysis in which we stratified the associations for child BMI by maternal BMI group (Table 5). This table shows similar associations in all the subgroups without strong evidence of a difference in effect estimates with maternal BMI. Adding maternal pre-pregnancy BMI and child BMI in the same model did not substantially change our reported associations for child BMI but did alter the associations for maternal BMI (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

The key finding of this study is that obesity parameters in children are associated with shorter 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 indicate the possibility of premature aging due to exposures in early childhood. This study is, to the best of our knowledge, the largest by far in assessing childhood obesity effects on telomere length.

Previous case-control studies investigating telomere length and childhood obesity have found conflicting results. In a study of 148 Arab children, mean telomere length was shorter in obese boys compared with lean boys [222], whereas, in a study of 53 Italian children no difference in telomere length was found between obese and non-obese individuals [220]. 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 [221]. All these studies were case-control design and so limited by selection bias.

In adults meta-analytical evidence suggests that leukocyte telomere length is inversely associated with BMI. [202]. In Chinese women, ages 40-70 years, BMI, waist circumference, and hip circumference were associated with shorter telomeres [230]. In a study encompassing 989 middle-aged individuals, a negative correlation was found between telomere length and obesity parameters [231]. 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 [232].Furthermore, a recent study of Martens et al. [179] 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 and metabolic 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 noteworthy. 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 [74, 233, 234]. 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 [74]. 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 accelerate shortening of telomeres and cellular replication [76, 235]. Oxidative stress is a direct and indirect source of single strand breaks in DNA [76]. Compared to genomic DNA, telomeres contain G-rich fragments that are highly sensitive to ROS and make them an ideal target for oxidative damage, and telomeric DNA is relatively less capable of DNA repair. Consequently, the higher level of oxidative stress induced by obesity leads to breakage of DNA and a more rapid decline in telomere length [191].

The telomere loss in childhood may lead to increased risk for chronic diseases in adulthood. We were not able to estimate the effects of telomere loss based on absolute telomere length, since we used a real-time PCR method that cannot provide these absolute values. Nevertheless, an estimation based on available data from young adulthood telomere length would suggest 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 leukocyte telomere length 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).

There are a number of potential limitations in our study. Firstly, we used a real-time PCR method to determine telomere length, which in general has a higher assay variability compared to the traditionally used TRF method [203, 204]. 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. 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 in our study as no data on maternal telomere length was available. Paternal age exerts a considerable effect on child telomere length [205], however, this data was not available in our cohorts. Finally, other potential important factors that occur during pregnancy and childhood, such as potential telomerase activity in children 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 comprehensive 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.

In conclusion, we have demonstrated that children with higher adiposity indicators have shorter telomeres in blood 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.

Funding

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 308333 – the HELIX project. Tim S. Nawrot was funded by the EU Program "Ideas" (ERC-2012-StG 310898).

Aknowledgements

ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya. We are grateful to all the participating families in the six countries who took part in this study.

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 (%).

CHAPTER 6

General Discussion

Aging is a complex physiological phenotype, responsive to a plethora of environmental stressors, including air pollution exposure and obesity, 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, 104]

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 and a schematic overview is presented in Figure 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: Prenatal air pollution, birth weight and mtDNA	• 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: Prenatal air pollution, infant growth and mtDNA	• 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: Air pollution exposure and telomeres	• 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: Obesity indicators and telomeres	• 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

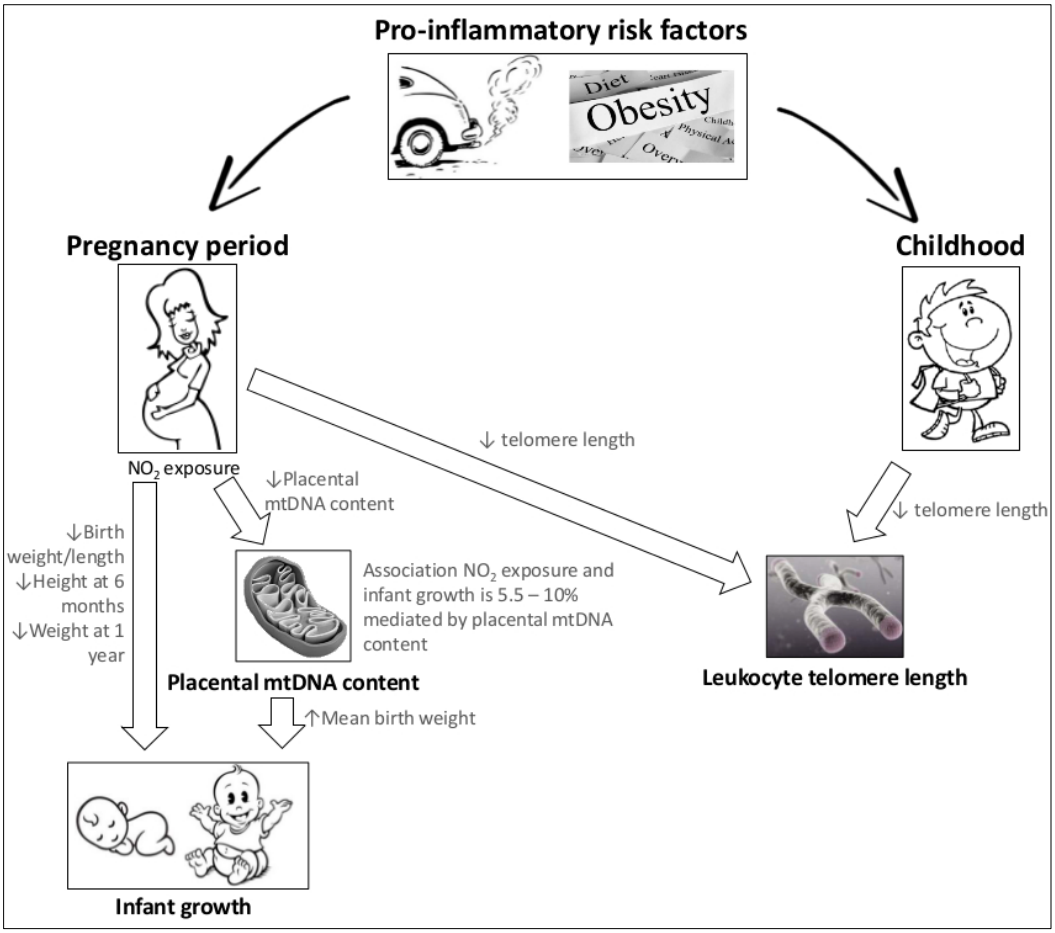

Figure 1. Schematic overview of this doctoral dissertation

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.[139] 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.[111, 236] 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, 237, 238] 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.[148] 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.[236] This barrier contains placental transporter that can block or facilitate foreign compounds.[110, 236] 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.[236] 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.[109]

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.[239] With advancing age, mutations and oxidative damage, mitochondrial DNA accumulates and causes a decrease in functionality.[240] 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 [82], a decrease [154, 155], and no change in mtDNA content [152] in adults and elderly. Short-to-moderate-term ambient black carbon levels [156] and benzene exposure [157] 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 [151, 158]. Although, recently a significant inverse association between prenatal $PM_{2.5}$ exposure and lower mtDNA content in cord blood was found [159].

As shown above, environmental exposures have been reported to result in both decreased and increased mtDNA content. This discrepancy in the mtDNA content results, can be explained by the very dynamic nature of mtDNA. MtDNA content 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.[160, 161] 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 [79]. 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 [162].

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.[89] 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.[58] Telomeres shorten after each cellular division due to the end-replication problem.[59] 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.[75] 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.[195] 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 [195]. 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.[194] 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.[155]

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.[197] 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.[193]

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.[155] Acute exposure to metal-rich PM exposure was positively associated with leukocyte telomere length in steel workers [241]. 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 [154, 155, 241].

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 [232]. In adult women, Valdes et al [217] 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 [231].

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.[125] Low birth weight has been associated with an increased risk of insulin-resistance syndrome [242], neurobehavioral problems [243], hypertension [244], cardiovascular [105, 245], metabolic [246], and renal disease [105] 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 [85, 152], and breast cancer [153].

Telomere length is considered as a marker of the biological aging process [59] 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 [247]. 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 [68, 169, 170], type 2 diabetes [171], cancer [248, 249], and increased mortality [172-174]. Furthermore, it is believed that the natural erosion of telomeres is accelerated through oxidative stress and inflammation [175].

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.[250, 251] 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.

The possibility of reverse causation is also an important limitation of observational studies. In epidemiology, reverse causation refers to a situation when the exposure is affected by the outcome. Reverse causation cannot be excluded in chapter 5 of this PhD project. We cannot rule out that telomere shortening is the causing factor of obesity in children.

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 [252]. 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 [253]. 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 [254]. 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 [254].

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.

Reference List

1. Barker, D.J.P., *FETAL ORIGINS OF CORONARY HEART-DISEASE.* British Medical Journal, 1995. 311(6998): p. 171-174.
2. Dietert, R.R., et al., *Breaking patterns of environmentally influenced disease for health risk reduction: immune perspectives.* Environ Health Perspect, 2010. 118(8): p. 1091-9.
3. Barker, D.J., et al., *Fetal nutrition and cardiovascular disease in adult life.* Lancet, 1993. 341(8850): p. 938-41.
4. WHO. *Health Risk of Particulate Matter from Long-range Transboundary Air Pollution* 2006 26/07/2017]; Available from: http://www.euro.who.int/__data/assets/pdf_file/0006/78657/E88189.pdf.
5. Brauer, M., et al., *A cohort study of traffic-related air pollution impacts on birth outcomes.* Environmental Health Perspectives, 2008. 116(5): p. 680-686.
6. Sunyer, J., et al., *Nitrogen dioxide is not associated with respiratory infection during the first year of life.* International Journal of Epidemiology, 2004. 33(1): p. 116-120.
7. Pope, C.A., 3rd and D.W. Dockery, *Health effects of fine particulate air pollution: lines that connect.* J Air Waste Manag Assoc, 2006. 56(6): p. 709-42.
8. Sioutas, C., R.J. Delfino, and M. Singh, *Exposure Assessment for Atmospheric Ultrafine Particles (UFPs) and Implications in Epidemiologic Research.* Environmental Health Perspectives, 2005. 113(8): p. 947-955.
9. Masri, S., C.M. Kang, and P. Koutrakis, *Composition and sources of fine and coarse particles collected during 2002-2010 in Boston, MA.* J Air Waste Manag Assoc, 2015. 65(3): p. 287-97.
10. Sillanpää, M., et al., *Chemical composition and mass closure of particulate matter at six urban sites in Europe.* Atmospheric Environment, 2006. 40: p. 212-223.
11. Brauer, M., et al., *Air pollution and retained particles in the lung.* Environmental Health Perspectives, 2001. 109(10): p. 1039-1043.
12. Kunihiko, H., et al., *Phagocytosis And Clearance Of Particulate Matter By Lung Macrophages: Effects Of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors (statins).* Environ Health Perspect, 2001: p. A27-A64.
13. Beelen, R., et al., *Long-term exposure to air pollution and cardiovascular mortality: an analysis of 22 European cohorts.* Epidemiology, 2014. 25(3): p. 368-78.
14. Hamra, G.B., et al., *Outdoor particulate matter exposure and lung cancer: a systematic review and meta-analysis.* Environ Health Perspect, 2014. 122(9): p. 906-11.
15. Laden, F., et al., *Reduction in fine particulate air pollution and mortality: Extended follow-up of the Harvard Six Cities study.* Am J Respir Crit Care Med, 2006. 173(6): p. 667-72.
16. Provost, E.B., et al., *Carotid intima-media thickness, a marker of subclinical atherosclerosis, and particulate air pollution exposure: the meta-analytical evidence.* PLoS One, 2015. 10(5): p. e0127014.
17. Brunekreef, B. and S. Holgate, *Air pollution and health.* Lancet, 2002. 360: p. 1233 - 1242.

18. Brook, R.D., et al., *Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association.* Circulation, 2010. 121(21): p. 2331-78.
19. Mills, N.L., et al., *Adverse cardiovascular effects of air pollution.* Nature Clinical Practice Cardiovascular Medicine, 2009. 6(1): p. 36-44.
20. Shah, P.S. and T. Balkhair, *Air pollution and birth outcomes: A systematic review.* Environment International, 2011. 37(2): p. 498-516.
21. Mehta, S., et al., *Ambient particulate air pollution and acute lower respiratory infections: a systematic review and implications for estimating the global burden of disease.* Air Quality, Atmosphere, & Health, 2013. 6(1): p. 69-83.
22. Anderson, J.O., J.G. Thundiyil, and A. Stolbach, *Clearing the Air: A Review of the Effects of Particulate Matter Air Pollution on Human Health.* Journal of Medical Toxicology, 2012. 8(2): p. 166-175.
23. Hoek, G., et al., *A review of land-use regression models to assess spatial variation of outdoor air pollution.* Atmospheric Environment, 2008. 42(33): p. 7561-7578.
24. Gotschi, T., et al., *Long-term effects of ambient air pollution on lung function: a review.* Epidemiology, 2008. 19(5): p. 690-701.
25. Gakidou, E., et al., *Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016.* The Lancet. 390(10100): p. 1345-1422.
26. Kim, J., *Ambient Air Pollution: Health Hazards to Children.* Pediatrics, 2004. 114(6): p. 1699.
27. Sram, R.J., et al., *Ambient air pollution and pregnancy outcomes: a review of the literature.* Environ Health Perspect, 2005. 113(4): p. 375-82.
28. Scheers, H., et al., *Does Air Pollution Trigger Infant Mortality in Western Europe? A Case-Crossover Study.* Environmental Health Perspectives, 2011. 119(7): p. 1017-1022.
29. Pedersen, M., et al., *Ambient air pollution and low birthweight: a European cohort study (ESCAPE).* The Lancet Respiratory Medicine, 2013. 1(9): p. 695-704.
30. Stieb, D.M., et al., *Ambient air pollution, birth weight and preterm birth: A systematic review and meta-analysis.* Environmental Research, 2012. 117(0): p. 100-111.
31. Bråbäck, L. and B. Forsberg, *Does traffic exhaust contribute to the development of asthma and allergic sensitization in children: findings from recent cohort studies.* Environmental Health, 2009. 8: p. 17-17.
32. Schembari, A., et al., *Ambient Air Pollution and Newborn Size and Adiposity at Birth: Differences by Maternal Ethnicity (the Born in Bradford Study Cohort).* Environ Health Perspect, 2015. 123(11): p. 1208-15.
33. Frutos, V., et al., *Impact of air pollution on fertility: a systematic review.* Gynecol Endocrinol, 2015. 31(1): p. 7-13.
34. Lamichhane, D., Leem, JH, Lee JY, Kim HC, *A meta-analysis of exposure to particulate matter and adverse birth outcomes.* 2015. 30: p. e2015011.

35. Sun, X., et al., *The association between fine particulate matter exposure during pregnancy and preterm birth: a meta-analysis.* BMC Pregnancy and Childbirth, 2015. 15(1): p. 300.
36. Sun, X., et al., *The associations between birth weight and exposure to fine particulate matter (PM2.5) and its chemical constituents during pregnancy: A meta-analysis.* Environ Pollut, 2016. 211: p. 38-47.
37. Ballester, F., et al., *Air pollution exposure during pregnancy and reduced birth size: a prospective birth cohort study in Valencia, Spain.* Environmental Health, 2010. 9.
38. Jedrychowski, W., et al., *Gender differences in fetal growth of newborns exposed prenatally to airborne fine particulate matter.* Environmental Research, 2009. 109(4): p. 447-456.
39. Slama, R., et al., *Maternal Personal Exposure to Airborne Benzene and Intrauterine Growth.* Environmental Health Perspectives, 2009. 117(8): p. 1313-1321.
40. Aguilera, I., et al., *Prenatal Exposure to Traffic-Related Air Pollution and Ultrasound Measures of Fetal Growth in the INMA Sabadell Cohort.* Environmental Health Perspectives, 2010. 118(5): p. 705-711.
41. Hansen, C.A., A.G. Barnett, and G. Pritchard, *The effect of ambient air pollution during early pregnancy on fetal ultrasonic measurements during mid-pregnancy.* Environmental Health Perspectives, 2008. 116(3): p. 362-369.
42. Bertin, M., et al., *Sex-specific differences in fetal growth in newborns exposed prenatally to traffic-related air pollution in the PELAGIE mother-child cohort (Brittany, France).* Environ Res, 2015. 142: p. 680-7.
43. Harris, G., et al., *The association of PM(2.5) with full term low birth weight at different spatial scales.* Environ Res, 2014. 134: p. 427-34.
44. Kim, E., et al., *Particulate matter and early childhood body weight.* Environ Int, 2016. 94: p. 591-9.
45. Jerrett, M., et al., *Traffic-related air pollution and obesity formation in children: a longitudinal, multilevel analysis.* Environ Health, 2014. 13: p. 49.
46. Wei, Y., et al., *Chronic exposure to air pollution particles increases the risk of obesity and metabolic syndrome: findings from a natural experiment in Beijing.* Faseb j, 2016. 30(6): p. 2115-22.
47. Iniguez, C., et al., *Prenatal Exposure to NO2 and Ultrasound Measures of Fetal Growth in the Spanish INMA Cohort.* Environ Health Perspect, 2016. 124(2): p. 235-42.
48. Risom, L., P. Moller, and S. Loft, *Oxidative stress-induced DNA damage by particulate air pollution.* Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 2005. 592(1-2): p. 119-137.
49. WHO. *Fact Sheet Obesity and Overweight.* 2016 August 30, 2017]; Available from: http://www.who.int/mediacenter/factsheets/fs311/en/.
50. de Onis, M., et al., *Development of a WHO growth reference for school-aged children and adolescents.* Bull World Health Organ, 2007. 85(9): p. 660-7.
51. Torloni, M.R., et al., *Prepregnancy BMI and the risk of gestational diabetes: a systematic review of the literature with meta-analysis.* Obes Rev, 2009. 10.
52. Furukawa, S., et al., *Increased oxidative stress in obesity and its impact on metabolic syndrome.* J Clin Invest, 2004. 114.

53. Popkin, B.M., L.S. Adair, and S.W. Ng, *Global nutrition transition and the pandemic of obesity in developing countries.* Nutr Rev, 2012. 70(1): p. 3-21.
54. Haidar, Y.M. and B.C. Cosman, *Obesity epidemiology.* Clin Colon Rectal Surg, 2011. 24(4): p. 205-10.
55. Halldorsson, T.I., et al., *Prenatal Exposure to Perfluorooctanoate and Risk of Overweight at 20 Years of Age: A Prospective Cohort Study.* Environmental Health Perspectives, 2012. 120(5): p. 668-673.
56. Factor-Litvak, P., et al., *Leukocyte Telomere Length in Newborns: Implications for the Role of Telomeres in Human Disease.* Pediatrics, 2016.
57. Aunan, J.R., et al., *Molecular and biological hallmarks of ageing.* Br J Surg, 2016. 103(2): p. e29-46.
58. Blackburn, E.H., *Structure and function of telomeres.* Nature, 1991. 350(6319): p. 569-573.
59. Levy, M.Z., et al., *Telomere end-replication problem and cell aging.* J Mol Biol, 1992. 225(4): p. 951-60.
60. Hayflick, L., *The limited in vitro lifetime of human diploid cell strains.* Experimental Cell Research, 1965. 37(3): p. 614-636.
61. Shay, J.W. and W.E. Wright, *Hayflick, his limit, and cellular ageing.* Nat Rev Mol Cell Biol, 2000. 1(1): p. 72-6.
62. Campisi, J. and F. d'Adda di Fagagna, *Cellular senescence: when bad things happen to good cells.* Nat Rev Mol Cell Biol, 2007. 8(9): p. 729-40.
63. Blackburn, E.H., E.S. Epel, and J. Lin, *Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection.* Science, 2015. 350(6265): p. 1193.
64. Kim, N.W., et al., *Specific association of human telomerase activity with immortal cells and cancer.* Science, 1994. 266(5193): p. 2011-5.
65. Hiyama, K., et al., *Activation of telomerase in human lymphocytes and hematopoietic progenitor cells.* J Immunol, 1995. 155(8): p. 3711-5.
66. O'Sullivan, R.J. and J. Karlseder, *Telomeres: protecting chromosomes against genome instability.* Nature reviews. Molecular cell biology, 2010. 11(3): p. 171-181.
67. Zhao, J., et al., *Association between Telomere Length and Type 2 Diabetes Mellitus: A Meta-Analysis.* PLOS ONE, 2013. 8(11): p. e79993.
68. Haycock, P.C., et al., *Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis.* Bmj, 2014. 349: p. g4227.
69. Andrew, T., et al., *Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs.* Am J Hum Genet, 2006. 78(3): p. 480-6.
70. Njajou, O.T., et al., *Shorter telomeres are associated with obesity and weight gain in the elderly.* Int J Obes (Lond), 2012. 36(9): p. 1176-9.
71. Okuda, K., et al., *Telomere length in the newborn.* Pediatr Res, 2002. 52.
72. Broer, L., et al., *Meta-analysis of telomere length in 19,713 subjects reveals high heritability, stronger maternal inheritance and a paternal age effect.* Eur J Hum Genet, 2013. 21(10): p. 1163-8.
73. Herbert, K.E., et al., *Angiotensin II-mediated oxidative DNA damage accelerates cellular senescence in cultured human vascular smooth*

muscle cells via telomere-dependent and independent pathways. Circ Res, 2008. 102(2): p. 201-8.
74. Minamino, T., et al., *A crucial role for adipose tissue p53 in the regulation of insulin resistance.* Nat Med, 2009. 15.
75. von Zglinicki, T., *Role of oxidative stress in telomere length regulation and replicative senescence.* Ann N Y Acad Sci, 2000. 908: p. 99-110.
76. von Zglinicki, T., *Oxidative stress shortens telomeres.* Trends Biochem Sci, 2002. 27(7): p. 339-44.
77. Clay Montier, L.L., Denk, J.J, Bai, Y., *Number matters: control of mammalian mitochondrial DNA copy number.* Journal of Genetics and Genomics, 2009. 36: p. 125-131.
78. Lamson, D.W. and S.M. Plaza, *Mitochondrial Factors in the Pathogenesis of Diabetes: A hypothesis for Treatment.* Alternative Medicine Review, 2002. 7(2): p. 94-111.
79. Lee, H.C. and Y.H. Wei, *Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress.* International Journal of Biochemistry & Cell Biology, 2005. 37(4): p. 822-834.
80. Robin, E.D. and R. Wong, *Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells.* J Cell Physiol, 1988. 136(3): p. 507-13.
81. Lee, H.C. and Y.H. Wei, *Mitochondrial role in life and death of the cell.* Journal of Biomedical Science, 2000. 7(1): p. 2-15.
82. Hou, L., et al., *Airborn particulate matter and mitochondrial damage: a cross-sectional study.* Environmental Health, 2010. 8(48).
83. Choi, Y.S., S. Kim, and Y.K. Pak, *Mitochondrial transcription factor A (mtTFA) and diabetes.* Diabetes Res Clin Pract, 2001. 54 Suppl 2: p. S3-9.
84. Wong, J., et al., *Mitochondrial DNA content in peripheral blood monocytes: relationship with age of diabetes onsetand diabetic complications.* Diabetologia, 2009. 52(9): p. 1953-1961.
85. Gianotti, T.F., et al., *A decreased mitochondrial DNA content is related to insulin resistance in adolescents.* Obesity, 2008. 16(7): p. 1591-1595.
86. Xia, P., et al., *Decreased mitochondrial DNA content in blood samples of patients with stage I breast cancer.* BMC Cancer, 2009. 9(1): p. 454.
87. Yu, M., et al., *Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients.* IUBMB Life, 2007. 59.
88. Blokhin, A., et al., *Variations in mitochondrial DNA copy numbers in MS brains.* Journal of Molecular Neuroscience, 2008. 35(3): p. 283-287.
89. Xing, J., et al., *Mitochondrial DNA content: Its genetic heritability and association with renal cell carcinoma.* Journal of the National Cancer Institute, 2008. 100(15): p. 1104-1112.
90. Ballinger, S.W., et al., *Mitochondrial integrity and function in atherogenesis.* Circulation, 2002. 106(5): p. 544-9.
91. Roy Chowdhury, S.K., et al., *Effects of extensively oxidized low-density lipoprotein on mitochondrial function and reactive oxygen species in porcine aortic endothelial cells.* American Journal of Physiology - Endocrinology And Metabolism, 2009. 298(1): p. E89.
92. Lynch, S.M., et al., *Mitochondrial DNA copy number and pancreatic cancer in the alpha-tocopherol beta-carotene cancer prevention study.* Cancer Prev Res (Phila), 2011. 4(11): p. 1912-9.

93. Dasgupta, S., et al., *Following mitochondrial footprints through a long mucosal path to lung cancer.* PLoS One, 2009. 4(8): p. e6533.
94. Lattuada, D., et al., *Higher mitochondrial DNA content in human IUGR placenta.* Placenta, 2008. 29(12): p. 1029-33.
95. Colleoni, F., et al., *Maternal blood mitochondrial DNA content during normal and intrauterine growth restricted (IUGR) pregnancy.* Am J Obstet Gynecol, 2010. 203(4): p. 365.e1-6.
96. Janssen, B.G., et al., *Cohort Profile: The ENVIRonmental influence ON early AGEing (ENVIRONAGE): a birth cohort study.* Int J Epidemiol, 2017.
97. Guxens, M., et al., *Cohort Profile: the INMA--INfancia y Medio Ambiente--(Environment and Childhood) Project.* Int J Epidemiol, 2012. 41(4): p. 930-40.
98. Wright, J., et al., *Cohort Profile: the Born in Bradford multi-ethnic family cohort study.* Int J Epidemiol, 2013. 42(4): p. 978-91.
99. Heude, B., et al., *Cohort Profile: The EDEN mother-child cohort on the prenatal and early postnatal determinants of child health and development.* Int J Epidemiol, 2016. 45(2): p. 353-63.
100. Grazuleviciene, R., *Maternal Smoking, GSTM1 and GSTT1 Polymorphism and Susceptibility to Adverse Pregnancy Outcomes.* 2009. 6(3): p. 1282-97.
101. Magnus, P., et al., *Cohort Profile Update: The Norwegian Mother and Child Cohort Study (MoBa).* Int J Epidemiol, 2016. 45(2): p. 382-8.
102. Chatzi, L., et al., *Metabolic syndrome in early pregnancy and risk of preterm birth.* Am J Epidemiol, 2009. 170(7): p. 829-36.
103. Vrijheid, M., et al., *The Human Early-Life Exposome (HELIX): Project Rationale and Design*. 2014.
104. Gluckman, P.D., et al., *Effect of in utero and early-life conditions on adult health and disease.* New England Journal of Medicine, 2008. 359(1): p. 61-73.
105. Risnes, K.R., et al., *Birthweight and mortality in adulthood: a systematic review and meta-analysis.* International Journal of Epidemiology, 2011. 40(3): p. 647-661.
106. Chahine, T., et al., *Particulate air pollution, oxidative stress genes, and heart rate variability in an elderly cohort.* Environmental Health Perspectives, 2007. 115(11): p. 1617-1622.
107. Li, N., T. Xia, and A.E. Nel, *The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles.* Free Radical Biology and Medicine, 2008. 44(9): p. 1689-1699.
108. Sahin, E., et al., *Telomere dysfunction induces metabolic and mitochondrial compromise.* Nature, 2011. 470(7334): p. 359-365.
109. Storvik, M., et al., *The unique characteristics of the placental transcriptome and the hormonal metabolism enzymes in placenta.* Reproductive Toxicology, 2014. 47(0): p. 9-14.
110. Myllynen, P., M. Pasanen, and O. Pelkonen, *Human placenta: a human organ for developmental toxicology research and biomonitoring.* Placenta, 2005. 26(5): p. 361-371.
111. Morello-Frosch, R., et al., *Ambient air pollution exposure and full-term birth weight in California.* Environmental Health, 2010. 9.

112. Gemma, C., et al., *Mitochondrial DNA depletion in small- and large-for-gestational-age newborns.* Obesity, 2006. 14(12): p. 2193-2199.
113. Janssen, B.G., et al., *Placental Mitochondrial DNA Content and Particulate Air Pollution during in Utero Life.* Environmental Health Perspectives, 2012. 120(9): p. 1346-1352.
114. Guxens, M., et al., *Prenatal Exposure to Residential Air Pollution and Infant Mental Development: Modulation by Antioxidants and Detoxification Factors.* Environmental Health Perspectives, 2012. 120(1): p. 144-149.
115. Cox, B., et al., *Impact of a stepwise introduction of smoke-free legislation on the rate of preterm births: analysis of routinely collected birth data*. Vol. 346. 2013.
116. Aguilera, I., et al., *Estimation of outdoor NOx, NO2, and BTEX exposure in a cohort of pregnant women using land use regression modeling.* Environmental Science & Technology, 2008. 42(3): p. 815-821.
117. Iniguez, C., et al., *Estimation of personal NO2 exposure in a cohort of pregnant women.* Science of the Total Environment, 2009. 407(23): p. 6093-6099.
118. Jacobs, L., et al., *Air Pollution-Related Prothrombotic Changes in Persons with Diabetes.* Environmental Health Perspectives, 2010. 118(2): p. 191-196.
119. Janssen, S., et al., *Spatial interpolation of air pollution measurements using CORINE land cover data.* Atmospheric Environment, 2008. 42(20): p. 4884-4903.
120. Lefebvre, W., et al., *Validation of the MIMOSA-AURORA-IFDM model chain for policy support: Modeling concentrations of elemental carbon in Flanders.* Atmospheric Environment, 2011. 45(37): p. 6705-6713.
121. Valeri, L. and T.J. VanderWeele, *Mediation Analysis Allowing for Exposure-Mediator Interactions and Causal Interpretation: Theoretical Assumptions and Implementation With SAS and SPSS Macros.* Psychological Methods, 2013. 18(2): p. 137-150.
122. Inanc, F., et al., *Relationship between oxidative stress in cord blood and route of delivery.* Fetal Diagnosis and Therapy, 2005. 20(5): p. 450-453.
123. Estarlich, M., et al., *Residential Exposure to Outdoor Air Pollution during Pregnancy and Anthropometric Measures at Birth in a Multicenter Cohort in Spain.* Environmental Health Perspectives, 2011. 119(9): p. 1333-1338.
124. Grandjean, P., et al., *The faroes statement: Human health effects of developmental exposure to chemicals in our environment.* Basic & Clinical Pharmacology & Toxicology, 2008. 102(2): p. 73-75.
125. Barker, D.J.P., *The developmental origins of adult disease.* Journal of the American College of Nutrition, 2004. 23(6): p. 588S-595S.
126. Hindmarsh, P.C., et al., *Factors predicting ante- and postnatal growth.* Pediatr Res, 2008. 63(1): p. 99-102.
127. Victora, C.G., et al., *Maternal and child undernutrition: consequences for adult health and human capital.* Lancet, 2008. 371(9609): p. 340-57.
128. Godfrey, K.M. and D.J. Barker, *Fetal nutrition and adult disease.* Am J Clin Nutr, 2000. 71(5 Suppl): p. 1344s-52s.
129. Olsen, J., et al., *The association of indicators of fetal growth with visual acuity and hearing among conscripts.* Epidemiology, 2001. 12(2): p. 235-8.

130. Li, N., et al., *Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage.* Environmental Health Perspectives, 2003. 111(4): p. 455-460.
131. Yan, W., et al., *Acute nitrogen dioxide inhalation induces mitochondrial dysfunction in rat brain.* Environmental Research, 2015. 138: p. 416-424.
132. Colicino, E., et al., *Mitochondrial haplogroups modify the effect of black carbon on age-related cognitive impairment.* Environmental Health, 2014. 13.
133. Meyer, J.N., et al., *Mitochondria as a Target of Environmental Toxicants.* Toxicological Sciences, 2013. 134(1): p. 1-17.
134. Clemente, D.B., et al., *Prenatal Ambient Air Pollution, Placental Mitochondrial DNA Content, and Birth Weight in the INMA (Spain) and ENVIRONAGE (Belgium) Birth Cohorts.* Environ Health Perspect, 2016. 124(5): p. 659-665.
135. Guxens, M., et al., *Cohort Profile: The INMA-INfancia y Medio Ambiente-(Environment and Childhood) Project.* International Journal of Epidemiology, 2012. 41(4): p. 930-940.
136. Hellemans, J., et al., *qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.* Genome Biology, 2007. 8(2): p. R19.
137. Valvi, D., et al., *Prenatal bisphenol a urine concentrations and early rapid growth and overweight risk in the offspring.* Epidemiology, 2013. 24(6): p. 791-9.
138. de Onis, M., et al., *[WHO growth standards for infants and young children].* Arch Pediatr, 2009. 16(1): p. 47-53.
139. Lacasana, M., A. Esplugues, and F. Ballester, *Exposure to ambient air pollution and prenatal and early childhood health effects.* Eur J Epidemiol, 2005. 20(2): p. 183-99.
140. Proietti, E., et al., *Air pollution during pregnancy and neonatal outcome: a review.* J Aerosol Med Pulm Drug Deliv, 2013. 26(1): p. 9-23.
141. Glinianaia, S.V., et al., *Particulate air pollution and fetal health: a systematic review of the epidemiologic evidence.* Epidemiology, 2004. 15(1): p. 36-45.
142. Sram, R.J., et al., *Ambient air pollution and pregnancy outcomes: A review of the literature.* Environmental Health Perspectives, 2005. 113(4): p. 375-382.
143. Maisonet, M., et al., *A review of the literature on the effects of ambient air pollution on fetal growth.* Environ Res, 2004. 95(1): p. 106-15.
144. Iniguez, C., et al., *Prenatal exposure to traffic-related air pollution and fetal growth in a cohort of pregnant women.* Occupational and Environmental Medicine, 2012. 69(10): p. 736-744.
145. Muraro, A.P., et al., *Effect of tobacco smoke exposure during pregnancy and preschool age on growth from birth to adolescence: a cohort study.* BMC Pediatr, 2014. 14: p. 99.
146. Fenercioglu, A.K., et al., *Impaired postnatal growth of infants prenatally exposed to cigarette smoking.* Tohoku J Exp Med, 2009. 218(3): p. 221-8.
147. Fleisch, A.F., et al., *Prenatal exposure to traffic pollution: associations with reduced fetal growth and rapid infant weight gain.* Epidemiology, 2015. 26(1): p. 43-50.

148. Kannan, S., et al., *Exposures to airborne particulate matter and adverse perinatal outcomes: A biologically plausible mechanistic framework for exploring potential effect modification by nutrition.* Environmental Health Perspectives, 2006. 114(11): p. 1636-1642.
149. Byun, H.-M. and A. Baccarelli, *Environmental exposure and mitochondrial epigenetics: study design and analytical challenges.* Human Genetics, 2014. 133(3): p. 247-257.
150. Linnane, A.W., et al., *Mitochondrial DNA mutations as an important contributor to ageing and degenerative diseases.* Lancet, 1989. 1(8639): p. 642-5.
151. Bouhours-Nouet, N., et al., *Maternal smoking is associated with mitochondrial DNA depletion and respiratory chain complex III deficiency in placenta.* American Journal of Physiology-Endocrinology and Metabolism, 2005. 288(1): p. E171-E177.
152. Xia, Y., et al., *Ambient air pollution, blood mitochondrial DNA copy number and telomere length in a panel of diabetes patients.* Inhal Toxicol, 2015: p. 1-7.
153. Yu, M., et al., *Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients.* Iubmb Life, 2007. 59(7): p. 450-457.
154. Hou, L., et al., *Inhalable particulate matter and mitochondrial DNA copy number in highly exposed individuals in Beijing, China: a repeated-measure study.* Part Fibre Toxicol, 2013. 10: p. 17.
155. Pieters, N., et al., *Biomolecular markers within the core axis of aging and particulate air pollution exposure in the elderly: a cross-sectional study.* Environ Health Perspect, 2016. 124.
156. Zhong, J., et al., *Traffic-Related Air Pollution, Blood Pressure, and Adaptive Response of Mitochondrial Abundance.* Circulation, 2016. 133(4): p. 378-87.
157. Shen, M., et al., *Association between mitochondrial DNA copy number, blood cell counts, and occupational benzene exposure.* Environmental and Molecular Mutagenesis, 2008. 49(6): p. 453-457.
158. Garrabou, G., et al., *Molecular basis of reduced birth weight in smoking pregnant women: mitochondrial dysfunction and apoptosis.* Addict Biol, 2016. 21(1): p. 159-70.
159. Rosa, M.J., et al., *Identifying sensitive windows for prenatal particulate air pollution exposure and mitochondrial DNA content in cord blood.* Environment International, 2017. 98: p. 198-203.
160. Shaughnessy, D.T., et al., *Mitochondria, energetics, epigenetics, and cellular responses to stress.* Environ Health Perspect, 2014. 122(12): p. 1271-8.
161. Castegna, A., V. Iacobazzi, and V. Infantino, *The mitochondrial side of epigenetics.* Physiol Genomics, 2015. 47(8): p. 299-307.
162. Lee, H.C., et al., *Aging- and smoking-associated alteration in the relative content of mitochondrial DNA in human lung.* FEBS Lett, 1998. 441(2): p. 292-6.
163. Martorell, R., *Results and implications of the INCAP follow-up study.* J Nutr, 1995. 125(4 Suppl): p. 1127s-1138s.
164. McCarthy, A., et al., *Birth weight; postnatal, infant, and childhood growth; and obesity in young adulthood: evidence from the Barry Caerphilly Growth Study.* Am J Clin Nutr, 2007. 86(4): p. 907-13.

165. Yuen, R.K.C., et al., *Human Placental-Specific Epipolymorphism and its Association with Adverse Pregnancy Outcomes.* Plos One, 2009. 4(10).
166. Jaffe, A.E. and R.A. Irizarry, *Accounting for cellular heterogeneity is critical in epigenome-wide association studies.* Genome Biology, 2014. 15(2).
167. Houseman, E.A., et al., *DNA methylation arrays as surrogate measures of cell mixture distribution.* Bmc Bioinformatics, 2012. 13.
168. WHO *Air quality guidelines: global update 2005: particulate matter, ozone, nitrogen dioxide and sulfur dioxide.* Available: http://www.euro.who.int/data/assets/pdf_file/0005/78638/E90038.pdf/ [accessed 19 December 2013], 2005.
169. Brouilette, S.W., et al., *Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study.* Lancet, 2007. 369(9556): p. 107-14.
170. Fitzpatrick, A.L., et al., *Leukocyte Telomere Length and Cardiovascular Disease in the Cardiovascular Health Study.* American Journal of Epidemiology, 2007. 165(1): p. 14-21.
171. Willeit, P., et al., *Leucocyte telomere length and risk of type 2 diabetes mellitus: new prospective cohort study and literature-based meta-analysis.* PLoS One, 2014. 9(11): p. e112483.
172. Cawthon, R.M., et al., *Association between telomere length in blood and mortality in people aged 60 years or older.* Lancet, 2003. 361(9355): p. 393-5.
173. Fitzpatrick, A.L., et al., *Leukocyte telomere length and mortality in the Cardiovascular Health Study.* J Gerontol A Biol Sci Med Sci, 2011. 66(4): p. 421-9.
174. Needham, B.L., et al., *Leukocyte telomere length and mortality in the National Health and Nutrition Examination Survey, 1999-2002.* Epidemiology, 2015. 26(4): p. 528-35.
175. von Zglinicki, T., et al., *Human cell senescence as a DNA damage response.* Mech Ageing Dev, 2005. 126(1): p. 111-7.
176. Aubert, G. and P.M. Lansdorp, *Telomeres and aging.* Physiol Rev, 2008. 88(2): p. 557-79.
177. Yamaguchi , H., et al., *Mutations in TERT, the Gene for Telomerase Reverse Transcriptase, in Aplastic Anemia.* New England Journal of Medicine, 2005. 352(14): p. 1413-1424.
178. Cawthon, R.M., *Telomere length measurement by a novel monochrome multiplex quantitative PCR method.* Nucleic Acids Res, 2009. 37(3): p. e21.
179. Martens, D.S., et al., *Maternal pre-pregnancy body mass index and newborn telomere length.* BMC Medicine, 2016. 14(1): p. 148.
180. Eeftens, M., et al., *Development of Land Use Regression models for PM(2.5), PM(2.5) absorbance, PM(10) and PM(coarse) in 20 European study areas; results of the ESCAPE project.* Environ Sci Technol, 2012. 46(20): p. 11195-205.
181. Beelen, R., et al., *Development of NO2 and NOx land use regression models for estimating air pollution exposure in 36 study areas in Europe - The ESCAPE project.* Atmospheric Environment, 2013. 72: p. 10-23.
182. Rahmalia, A., et al., *Pregnancy exposure to atmospheric pollutants and placental weight: an approach relying on a dispersion model.* Environ Int, 2012. 48: p. 47-55.

183. Wang, M., et al., *Performance of multi-city land use regression models for nitrogen dioxide and fine particles.* Environ Health Perspect, 2014. 122(8): p. 843-9.
184. Beelen, R., et al., *Effects of long-term exposure to air pollution on natural-cause mortality: an analysis of 22 European cohorts within the multicentre ESCAPE project.* The Lancet, 2014. 383(9919): p. 785-795.
185. Nawrot, T.S., et al., *Public health importance of triggers of myocardial infarction: a comparative risk assessment.* The Lancet, 2011. 377(9767): p. 732-740.
186. Nafstad, P., et al., *Urban air pollution and mortality in a cohort of Norwegian men.* Environ Health Perspect, 2004. 112(5): p. 610-5.
187. Finkelstein, M.M., M. Jerrett, and M.R. Sears, *Traffic air pollution and mortality rate advancement periods.* Am J Epidemiol, 2004. 160(2): p. 173-7.
188. Pope , C.A.I., M. Ezzati , and D.W. Dockery *Fine-Particulate Air Pollution and Life Expectancy in the United States.* New England Journal of Medicine, 2009. 360(4): p. 376-386.
189. Epel, E.S., et al., *Cell aging in relation to stress arousal and cardiovascular disease risk factors.* Psychoneuroendocrinology, 2006. 31.
190. Grahame, T.J. and R.B. Schlesinger, *Oxidative stress-induced telomeric erosion as a mechanism underlying airborne particulate matter-related cardiovascular disease*
Particle and Fibre Toxicology, 2012. 9(21).
191. Kawanishi, S. and S. Oikawa, *Mechanism of telomere shortening by oxidative stress.* Ann N Y Acad Sci, 2004. 1019: p. 278-84.
192. Bijnens, E., et al., *Lower placental telomere length may be attributed to maternal residential traffic exposure; a twin study.* Environment International, 2015. 79(0): p. 1-7.
193. Martens, D.S., et al., *Prenatal Air Pollution and Newborns' Predisposition to Accelerated Biological Aging.* JAMA Pediatr, 2017.
194. McCracken, J., et al., *Annual ambient black carbon associated with shorter telomeres in elderly men: Veterans Affairs Normative Aging Study.* Environ Health Perspect, 2010. 118(11): p. 1564-70.
195. Hoxha, M., et al., *Association between leukocyte telomere shortening and exposure to traffic pollution: a cross-sectional study on traffic officers and indoor office workers.* Environ Health, 2009. 8: p. 41.
196. Hou, L., et al., *Air pollution exposure and telomere length in highly exposed subjects in Beijing, China: a repeated-measure study.* Environ Int, 2012. 48: p. 71-7.
197. Walton, R.T., et al., *Air pollution, ethnicity and telomere length in east London schoolchildren: An observational study.* Environ Int, 2016. 96: p. 41-47.
198. Hodes, R.J., K.S. Hathcock, and N.-p. Weng, *Telomeres in T and B cells.* Nat Rev Immunol, 2002. 2(9): p. 699-706.
199. Weng, N.-p., L. Granger, and R.J. Hodes, *Telomere lengthening and telomerase activation during human B cell differentiation.* Proceedings of the National Academy of Sciences of the United States of America, 1997. 94(20): p. 10827-10832.
200. Ward-Caviness, C.K., et al., *Long-term exposure to air pollution is associated with biological aging.* Oncotarget, 2016.

201. Nawrot, T.S., et al., *Telomere length and possible link to X chromosome.* The Lancet, 2004. 363(9408): p. 507-510.
202. Muezzinler, A., A.K. Zaineddin, and H. Brenner, *A systematic review of leukocyte telomere length and age in adults.* Ageing Res Rev, 2013. 12.
203. Aviv, A., et al., *Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR.* Nucleic Acids Res, 2011. 39.
204. Kimura, M., et al., *Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths.* Nat Protoc, 2010. 5.
205. Kimura, M., et al., *Offspring's leukocyte telomere length, paternal age, and telomere elongation in sperm.* PLoS Genet, 2008. 4(2): p. e37.
206. Ng, M., et al., *Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013.* Lancet, 2014. 384(9945): p. 766-81.
207. Caballero, B., *The global epidemic of obesity: an overview.* Epidemiol Rev, 2007. 29: p. 1-5.
208. Rankin, J.W., et al., *Effect of raisin consumption on oxidative stress and inflammation in obesity.* Diabetes Obes Metab, 2008. 10(11): p. 1086-96.
209. Suzuki, K., et al., *Relationship between obesity and serum markers of oxidative stress and inflammation in Japanese.* Asian Pac J Cancer Prev, 2003. 4(3): p. 259-66.
210. von Zglinicki, T., et al., *Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence?* Exp Cell Res, 1995. 220(1): p. 186-93.
211. Biron-Shental, T., et al., *Telomeres are shorter in placental trophoblasts of pregnancies complicated with intrauterine growth restriction (IUGR).* Early Human Development, 2010. 86(7): p. 451-456.
212. Lin, S., et al., *Short Placental Telomere was Associated with Cadmium Pollution in an Electronic Waste Recycling Town in China.* PLoS ONE, 2013. 8(4): p. e60815.
213. Hjelmborg, J.B., et al., *The heritability of leucocyte telomere length dynamics.* J Med Genet, 2015. 52.
214. Honig, L.S., et al., *Heritability of telomere length in a study of long-lived families.* Neurobiol Aging, 2015. 36.
215. Slagboom, P.E., S. Droog, and D.I. Boomsma, *Genetic determination of telomere size in humans: a twin study of three age groups.* Am J Hum Genet, 1994. 55.
216. Martens, D.S. and T.S. Nawrot, *Air pollution stress and the aging phenotype: the telomere connection.* Curr Environ Health Rep, 2016. 3.
217. Valdes, A.M., et al., *Obesity, cigarette smoking, and telomere length in women.* Lancet, 2005. 366.
218. Epel, E.S., et al., *Accelerated telomere shortening in response to life stress.* Proc Natl Acad Sci U S A, 2004. 101(49): p. 17312-5.
219. Kim, S., et al., *Obesity and weight gain in adulthood and telomere length.* Cancer Epidemiol Biomarkers Prev, 2009. 18(3): p. 816-20.
220. Zannolli, R., et al., *Telomere length and obesity.* Acta Paediatr, 2008. 97(7): p. 952-4.

221. Buxton, J.L., et al., *Childhood obesity is associated with shorter leukocyte telomere length.* J Clin Endocrinol Metab, 2011. 96(5): p. 1500-5.
222. Al-Attas, O.S., et al., *Telomere length in relation to insulin resistance, inflammation and obesity among Arab youth.* Acta Paediatr, 2010. 99(6): p. 896-9.
223. Entringer, S., et al., *Maternal psychosocial stress during pregnancy is associated with newborn leukocyte telomere length.* American Journal of Obstetrics and Gynecology, 2013. 208(2): p. 134.e1-134.e7.
224. Marchetto NM, G.R., Ferry ML, Ostojic M, Wolff SM, Yao R, Haussmann MF. , *Prenatal stress and newborn telomere length.* . 2016, Am J Obstet Gynecol. . p. e1–8.
225. Salihu HM, P.A., King L, Paothong A, Nwoga C, Marty PJ, Whiteman V., *Impact of intrauterine tobacco exposure on fetal telomere length.* . 2015, Am J Obstet Gynecol. . p. e1–8.
226. Wojcicki, J.M., et al., *Cord blood telomere length in Latino infants: relation with maternal education and infant sex.* J Perinatol, 2016. 36.
227. Biron-Shental, T., et al., *Telomeres are shorter in placentas from pregnancies with uncontrolled diabetes.* Placenta, 2015. 36.
228. Chatzi, L., et al., *Cohort Profile: The Mother-Child Cohort in Crete, Greece (Rhea Study).* Int J Epidemiol, 2017. 46(5): p. 1392-1393k.
229. Eurostat, *International Standard Classification of Education (ISCED)*.
230. Cui, Y., et al., *Associations of leukocyte telomere length with body anthropometric indices and weight change in Chinese women.* Obesity (Silver Spring), 2013. 21(12): p. 2582-8.
231. Nordfjall, K., et al., *Telomere length is associated with obesity parameters but with a gender difference.* Obesity (Silver Spring), 2008. 16(12): p. 2682-9.
232. Lee, M., et al., *Inverse association between adiposity and telomere length: The Fels Longitudinal Study.* Am J Hum Biol, 2011. 23(1): p. 100-6.
233. Despres, J.P. and I. Lemieux, *Abdominal obesity and metabolic syndrome.* Nature, 2006. 444(7121): p. 881-7.
234. Trayhurn, P. and I.S. Wood, *Adipokines: inflammation and the pleiotropic role of white adipose tissue.* Br J Nutr, 2004. 92(3): p. 347-55.
235. Saretzki, G. and T. Zglinicki, *Replicative aging, telomeres, and oxidative stress.* Ann N Y Acad Sci, 2002. 959.
236. Wick, P., et al., *Barrier Capacity of Human Placenta for Nanosized Materials.* Environmental Health Perspectives, 2010. 118(3): p. 432-436.
237. Dadvand, P., et al., *Maternal Exposure to Particulate Air Pollution and Term Birth Weight: A Multi-Country Evaluation of Effect and Heterogeneity.* Environmental Health Perspectives, 2013. 121(3): p. 367-373.
238. Sapkota, A., et al., *Exposure to particulate matter and adverse birth outcomes: a comprehensive review and meta-analysis.* Air Quality, Atmosphere & Health, 2012. 5(4): p. 369-381.
239. Liu, P. and B. Demple, *DNA repair in mammalian mitochondria: Much more than we thought?* Environ Mol Mutagen, 2010. 51(5): p. 417-26.
240. Chistiakov, D.A., et al., *Mitochondrial aging and age-related dysfunction of mitochondria.* Biomed Res Int, 2014. 2014: p. 238463.

241. Dioni, L., et al., *Effects of short-term exposure to inhalable particulate matter on telomere length, telomerase expression, and telomerase methylation in steel workers.* Environ Health Perspect, 2011. 119(5): p. 622-7.
242. Levy-Marchal, C. and D. Jaquet, *Long-term metabolic consequences of being born small for gestational age.* Pediatr Diabetes, 2004. 5(3): p. 147-53.
243. Aarnoudse-Moens, C.S., et al., *Meta-analysis of neurobehavioral outcomes in very preterm and/or very low birth weight children.* Pediatrics, 2009. 124(2): p. 717-28.
244. Mu, M., et al., *Birth weight and subsequent blood pressure: a meta-analysis.* Arch Cardiovasc Dis, 2012. 105(2): p. 99-113.
245. Huxley, R., et al., *Is birth weight a risk factor for ischemic heart disease in later life?* Am J Clin Nutr, 2007. 85(5): p. 1244-50.
246. Harder, T., et al., *Birth weight and subsequent risk of type 2 diabetes: a meta-analysis.* Am J Epidemiol, 2007. 165(8): p. 849-57.
247. Benetos, A., et al., *Tracking and fixed ranking of leukocyte telomere length across the adult life course.* Aging Cell, 2013. 12(4): p. 615-21.
248. Wentzensen, I.M., et al., *The Association of Telomere Length and Cancer: A Meta-Analysis.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2011. 20(6): p. 1238-1250.
249. Ma, H., et al., *Shortened telomere length is associated with increased risk of cancer: a meta-analysis.* PLoS One, 2011. 6(6): p. e20466.
250. Miettinen, O.S. and E.F. Cook, *Confounding: essence and detection.* Am J Epidemiol, 1981. 114(4): p. 593-603.
251. Greenland, S., J. Pearl, and J.M. Robins, *Causal diagrams for epidemiologic research.* Epidemiology, 1999. 10(1): p. 37-48.
252. EC, *European Commission Staff Working Document* 2013.
253. OECD, *The cost of air pollution. Health impacts of road transport. .* OECD Publishing, 2014.
254. Wolff, H. and L. Perry, *Policy MonitorTrends in Clean Air Legislation in Europe: Particulate Matter and Low Emission Zones1.* Review of Environmental Economics and Policy, 2010. 4(2): p. 293-308.

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)

12. **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)

13. **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. Young Research Conference on Environmental Epidemiology, ISEE, Barcelona, Spain (Poster presentation)

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)

Dankwoord

Het is moeilijk om met woorden te omschrijven wat voor een fantastisch avontuur mijn doctoraat is geweest. Een doctoraal maak je echter niet alleen. Daarom wil ik mij hier richten tot de fantastische groep mensen die hebben bijgedragen aan het tot stand komen van deze thesis.

Om te beginnen wil ik mijn promotoren, Prof. Dr Tim Nawrot en Prof. Dr Martine Vrijheid bedanken om mij de kans te geven dit doctoraat te starten. Tim, tijdens jou lessen heb ik de wereld van de epidemiologie leren kennen. Martine, dankzij jou heb ik de kans gekregen om een ongelofelijk leerzame buitenlandse ervaring op te doen. Ik wil jullie beide bedanken voor jullie begeleiding en voor wat ik van jullie heb geleerd tijdens mijn doctoraat. Het was fijn om samen te werken met personen die hun werk met zoveel passie en gedrevenheid aanpakken.

I would like to thank all the members of the jury for their critical evaluation of my PhD thesis: Prof. dr Marcel Ameloot, Prof. Dr Jordi Sunyer, Prof. Dr Veerle Somers, Prof. Dr Remy Slama, Prof. Dr Matty Weijenberg, Dr Karen Vrijens, and Dr Lidia Casas.

Ik wil de collega's van 'groep Nawrot' bedanken voor de uitzonderlijk aangename werksfeer. Naast werken, was er ook altijd wel tijd voor koffiepauzes en een leuke babbel ☺ Tijdens de congressen hebben we elkaar pas echt leren kennen en een aantal memorabele momenten beleefd: van een poging tot Spaans spreken "wijos kunnos goedos sprekos Spaanos", en cocktails drinken en salsa dansen met rode rozen in Barcelona, naar dansen en zingen voor het colosseum, tot belanden in een schunnig hotel…

Kantoor c107b, wij waren het kantoor die de oorzaak was van de verdubbeling van het aantal doctoraatsstudenten in 'groep Nawrot'. Wat was het fijn om met jullie een kantoor te delen! Narjes, jij was de "moederkloek" van ons bureau. Zo zorgzaam, zo lief en je stond ook altijd voor ons klaar. En niet te vergeten, jij hebt ook de magische kast geïntroduceerde. Honger lijden konden we nooit :P Dries, na de duizenden DNA extracties die we samen hebben uitgevoerd zijn we ondertussen al echte professionals. Bedankt om mij altijd met raad en daad bij te staan. Maria, it was so nice to talk to you about the amazing Greece, especially their food. The pictures and videos of the very cute Dimitris were always a good solution to cheer me up ☺ Ellen, wat heb ik jou de laatste maanden gemist! Hoewel ik 2 jaar in Barcelona heb gewerkt, kon ik altijd bij jou

terecht voor statistische hulp. Bedankt om altijd voor mij klaar te staan! Geniet nog van je maandje vrij met je prachtig dochtertje Estelle! Annette, wij hadden altijd wel iets om over te praten, het maakte niet uit als het nu over handtassen, schoenen of onze huisdieren ging. Mijn werkdag begon altijd een heel stuk beter na deze leuke gesprekken.

Nu komen we bij de 'oorspronkelijke groep Nawrot'. Bram, Nelly, Esmée en Nicky, jullie waren mijn begeleiders tijdens mijn juniorstage en/of seniorstage. Wat heb ik veel van jullie geleerd! Tijdens mijn doctoraat hebben we samen heel veel plezier beleefd. Nicky, het is altijd fijn om je nog eens terug te zien en bij te praten. Ik ben trouwens nog altijd op je housewarming aan het wachten :P Eline, het is al bijna een jaar geleden sinds je uitgevlogen bent naar je nieuwe job. Wat is de tijd voorbij gevlogen! Je wordt hier nog altijd gemist. Nog veel succes met je thesis! Michal, bedankt voor je statistische hulp in het begin van mijn doctoraat. Bianca, bedankt om altijd klaar te staan wanneer ik nood had om te ventileren. Ik ben blij dat ik je vorige zomer de weg heb kunnen wijzen in Barcelona :P Karen, bedankt voor de fijne gesprekken, je begeleiding en voor je raad doorheen de jaren.

Leen, jij begon net aan je doctoraat toen ik naar Barcelona verhuisde, waardoor we elkaar niet zo vaak hebben gezien. Wanneer we dan eens samen waren, stond je altijd klaar om samen even te lachen (geweldig om een collega te hebben met dezelfde sarcastische humor). Martien, de zomer van 2013 ga ik nooit meer vergeten. Samen zijn we erin geslaagd om honderden extracties uit te voeren in een heet labo tijdens een hete zomer. Bedankt voor de fijne babbels tijdens onze vele labomomenten samen. Verder wil ik Harry, Michelle, Janneke en Evi enorm dankbaar voor hun adviezen. Ik wil ook nog graag even de 'nieuwelingen' aan bod laten komen – Kristof, Yinthe, Katrien, Charlotte, Rosella en Hanne. Jullie hebben weer nieuw leven geblazen in 'groep Nawrot'! Ik wil ook nog alle andere collega's van het CMK bedanken voor de leuke middagpauzes.

A special thanks goes to my chicas in sala C. You girls made me feel more than welcome and at home in Barcelona. I can't imagine what I would have done without you! We've had some very nice times: going from calçotadas, to evenings of sardines i rumba, to a weekend at Tossa de Mar. Alba, it was

always a lot of fun around you! Deborah, wat was het 'zalig' om iemand te hebben om Nederlands mee te spreken, hoewel we elkaar niet altijd even goed verstonden (nu weet je ondertussen wel wat een kleedje is :P). Angela, thanks to you I know a lot of sayings in Spanish that I still use ☺ Èrica, thanks for always being there for me! I can't wait meeting Quim! Ariadna, it's always nice to have a friend that loves cava as much as me ☺ Girls, thanks to you, I have a new family! Furthermore, I want to thank Javier, Alejandro and Carlos for the very nice talks during lunch. I also want to thank the whole CREAL-ISGlobal family to make me feel more than welcome.

Ik wil ook nog graag al mijn vrienden bedanken voor de steun de afgelopen jaren. In het bijzonder Debora en Sarah. Debora, wij zijn ondertussen al 25 jaar bevriend en hebben altijd op elkaar kunnen rekenen. Ook al waren we vaak niet in staat om elkaar supervaak te zien, we stonden wel altijd klaar voor elkaar. Sarah, samen hebben we al heel wat avonturen beleefd! De vele vakanties samen zijn onvergetelijk. Bedankt voor al je bezoekjes in Barcelona, het was altijd even fijn om je terug te zien. En ook een heel dikke merci om mij te helpen met mijn (veel te veel) spullen te verhuizen van Barcelona terug naar België ☺ Esther, Sophie en Eveline, wat heb ik toch veel gehad aan jullie de laatste jaren! Als ik ontspanning nodig had, zat ik bij jullie aan het juiste adres. Van eens lekker gaan eten, naar een bezoekje aan Disneyland tot een safari in eigen land. Laat die andere reisjes ook nog maar komen! ☺ Sophie, samen zijn we bijna 10 jaar geleden aan ons avontuur aan de unief begonnen en nu is het bijna tijd om deze af te ronden. We konden onze frustraties en geluk altijd bij elkaar kwijt. Dank je om altijd voor mij klaar te staan!

Tot slot wil ik nog de familie Batalha en familie Paulussen bedanken die mij de voorbije jaren hebben gesteund. Dario en Katjana, bedankt om er altijd voor mij te zijn. Jullie hebben mij 2 memorabele momenten bezorgd tijdens mijn doctoraat: jullie trouw en niet te vergeten de geboorte van mijn overdreven schattig en lief metekindje Ebor. Mama en papa, ik zou de stap richting Barcelona nooit hebben kunnen zetten zonder jullie. Heel hard bedankt voor jullie hulp in mijn verhuis naar Barcelona. Maar vooral bedankt voor de kansen die jullie mij gegeven hebben en om altijd in mij te hebben geloofd. Dankzij al jullie steun heb ik zelfvertrouwen gekregen wat uiteindelijk heeft geresulteerd in de persoon die ik nu ben geworden.