PhD thesis presented on the 19th of September 2018 at Hasselt University

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Summary

According to the World Health Organization, nearly one in four of total global deaths is due to modifiable environmental stressors. Even before birth environmental exposures influence the onset and progression of chronic diseases later in life, which is referred to as the "Barker" or "Developmental Origins of Health and Disease" (DOHaD) hypothesis. Among the most harmful environmental risk factors are ambient particulate matter (PM) exposure and excess body fat. In Belgium, almost half of the population suffers from overweight and around 14% is obese.

Since the mechanisms driving environmental exposure-induced adverse health effects are poorly understood, in this doctoral dissertation, we tried to further elucidate underlying biochemical pathways by means of hypothesis-driven and hypothesis-generating approaches. We focused on mitochondrial DNA (mtDNA) content and telomere length, two molecular biomarkers of oxidative stress and inflammation. We furthermore implemented transcriptomic analyses to create new hypotheses concerning a biologically plausible link between PM exposure and adverse health effects.

The specific objectives of this doctoral dissertation were:

1. To investigate the effect of maternal PM_{10} exposure during pregnancy on birth weight and small-for-gestational-age
2. To assess sex-specific transcriptomic responses to particulate air pollution exposure in newborns and in adults
3. To explore the sex-specific cross-sectional and longitudinal association of peripheral blood telomere length and mtDNA content with two obesity measures in a general adult population
4. To analyze the association between peripheral blood mtDNA content and long-term $PM_{2.5}$ exposure in a general adult population

A summary of each chapter is shown in Table 1. Findings of this PhD project contribute to the international research investigating the impact of excess body fat and the impact of ambient PM at current exposure levels on human health. Promoting healthy weight by encouraging healthy food and physical activity in daily life, and lowering PM exposure below the WHO guidelines will help to reduce chronic diseases and mortality rates worldwide.

Table 1. Summary of this doctoral dissertation

Chapter	Background	What this study adds	Conclusions and perspectives

Chapter 2	• Reduced fetal growth is associated with adverse health effects later in life
	• Evidence for air pollution-associated effects on fetal growth is cumulating
	  ○ Inconsistency between study results regarding effect size, exposure window, …
		• Among neonates born after 31 weeks of gestation, maternal PM_{10} exposure is inversely associated with fetal growth
		  ○ Most pronounced effects in moderately preterm babies (32-36 weeks)
		• Segmented regression analysis showed strongest effects at lower PM_{10} levels
			• Even below current European air quality standards, prenatal PM_{10} exposure reduces birth weight and increases the risk of being small for gestational age

Chapter 3	• Prenatal PM exposure is associated with
	  ○ reduced fetal growth and adverse birth outcomes
	  ○ adverse health effects later in life
	• Underlying molecular mechanisms largely unknown
	• Sex-specific PM responses are reported by previous studies
	• The impact of prenatal PM exposure on fetal transcriptome profiles has not been assessed so far
		• Gene expression of DNA damage and olfactory signalling pathways were altered by respectively long- and short-term $PM_{2.5}$ exposure in both male and female newborns
		• For long-term exposure in boys neurodevelopment pathways were modulated, while in girls defensin expression was down-regulated.
		• For short-term exposure we identified pathways related to the mitochondrial function (boys) and immune response (girls)
			• This is the first whole genome mRNA expression study in cord blood to identify sex-specific pathways altered by $PM_{2.5}$ exposure
			• The identified transcriptome pathways could provide new molecular insights as to the interaction pattern of early life $PM_{2.5}$ exposure with the biological development of the fetus

Chapter 4	• Mitochondria are the main producers and targets of reactive oxygen species (ROS) • PM exposure has been linked with altered ROS production and mitochondrial functioning	• Sex-specific transcriptome analyses demonstrated that PM_{10} exposure is associated with mitochondrial genome maintenance and apoptosis for short-term exposure and to the electron transport chain (ETC) for medium-term exposure in women	• We identified mitochondrial genes and pathways associated with particulate air pollution showing upregulation of energy producing pathways as a potential mechanism to compensate for PM-induced mitochondrial damage

Table 1. Summary of this doctoral dissertation (continued)

Chapter	Background	What this study adds	Conclusions and perspectives
	• Both PM exposure and mitochondrial damage are associated with several chronic diseases • Studies investigating the impact of PM exposure on mitochondria-linked genes are scarce	• For men, medium-term PM_{10} exposure was associated with the tricarboxylic acid cycle • In a distinct study population, we were able to validate the association between PM_{10} exposure and expression of several ETC genes	
Chapter 5	• Telomere length and mtDNA content are ○ sensitive to oxidative stress and inflammation ○ implicated in the onset and progression of chronic diseases • The effect of overweight and obesity on telomere length and mtDNA content is unclear	• Telomere length was inversely associated with waist circumference • 4-year change in telomere length was inversely associated with change in waist circumference • A positive trend between 4-year change in mtDNA and waist circumference in men and a curvilinear association in women	• Our findings support the idea that restricting weight gain during aging may decelerate telomere shortening and affect mtDNA changes over time, especially in women
Chapter 6	• MtDNA content is a molecular biomarker of oxidative stress, inflammation, and mitochondrial damage • MtDNA content has been associated with PM exposure ○ results were inconsistent with respect to the direction of the effects	• Long-term $PM_{2.5}$ exposure was inversely associated with peripheral blood mtDNA content, especially in abdominal obese participants • Long-term $PM_{2.5}$ exposure was inversely associated with change in peripheral blood mtDNA content over a follow-up period of ±4 years ○ Effects more pronounced in smokers and participants with increasing waist circumference over follow-up period	• Even below the European air pollution guidelines, PM exposure affects mtDNA content • Future studies should take into account possible effect modification by (abdominal) obesity measures and potential synergism between smoking and environmental air pollution exposure

SAMENVATTING

Volgens de Wereldgezondheidsorganisatie is bijna een kwart van het aantal sterfgevallen wereldwijd te wijten aan omgevingsfactoren. Al voor de geboorte hebben omgevingsblootstellingen een impact op de ontwikkeling en progressie van chronische ziekten later in het leven, ook wel de "Barker" of "DOHaD" hypothese genoemd. Blootstelling aan luchtvervuiling en overgewicht behoren tot de meest schadelijke risicofactoren voor ouderdomsgerelateerde ziektes. In België lijdt bijna de helft van de bevolking aan overgewicht en ongeveer 14% is obees.

Aangezien de intermediaire link tussen omgevingsfactoren en nadelige gezondheidseffecten nog niet goed begrepen is, hebben we in dit proefschrift verder onderzoek gedaan naar mogelijke onderliggende biochemische processen met behulp van hypothesegedreven en hypothesegenererende methoden. We hebben ons gericht op mitochondriën en telomeerlengte, twee moleculaire biosensors van oxidatieve stress en ontsteking. Daarnaast hebben we transcriptoomwijde analyses uitgevoerd om nieuwe hypotheses te creëren met betrekking tot de link tussen fijnstofblootstelling en gezondheidscomplicaties.

De specifieke doelstellingen van dit proefschrift zijn:

1. Onderzoek naar het effect van prenatale fijnstofblootstelling op het geboortegewicht en het risico om geboren te worden met een te laag geboortegewicht voor de zwangerschapsduur
2. Analyseren van het effect van fijnstofblootstelling op het transcriptoom
3. Bestuderen van de geslachtsspecifieke cross-sectionele en longitudinale associatie van telomeerlengte en mitochondriaal DNA (mtDNA)-inhoud met overtollig lichaamsgewicht in een algemene volwassen populatie
4. Het verband analyseren tussen perifeer bloed mtDNA-inhoud en langdurige fijnstofblootstelling in een algemene volwassen populatie

Een samenvatting van elk hoofdstuk wordt getoond in Tabel 1. Bevindingen van dit doctoraatsonderzoek dragen bij aan het internationale onderzoek naar de impact van overtollig lichaamsvet en luchtvervuiling op de gezondheid. Het promoten van een normaal lichaamsgewicht door gezond te eten en het inplannen van fysieke activiteit in het dagelijks leven, en het verlagen van de blootstelling aan fijnstof tot onder de richtlijnen van de Wereldgezondheidsorganisatie, kunnen bijdragen tot het stabiliseren van de chronische ziekte-epidemie.

Tabel 1. Samenvatting van dit proefschrift

Hoofdstuk	Achtergrondinfomatie	Wat deze studie bijbrengt	Conclusies en perspectieven
Hoofdstuk 2	• Verschillende studies hebben aanwijzingen gevonden dat luchtvervuilingsblootstelling foetale groei vermindert ○ Inconsistenties tussen studieresultaten met betrekking tot effectgrootte, blootstellingsvenster, …	• Bij baby's geboren na 31 weken zwangerschap is prenatale fijnstofblootstelling negatief geassocieerd met de groei van de foetus ○ Meest uitgesproken effecten bij matig premature baby's (32-36 weken) • Breekpunt analyse toonde de sterkste effecten bij lagere fijnstofconcentraties	• Zelfs onder de huidige Europese luchtkwaliteitsnormen vermindert prenatale fijnstofblootstelling het geboortegewicht en verhoogt het het risico om geboren te worden met een te laag geboortegewicht voor de zwangerschapsduur
Hoofdstuk 3	• Prenatale fijnstofblootstelling is geassocieerd met ○ verminderde foetale groei en ongunstige geboorte-uitkomsten ○ nadelige gezondheidseffecten later in het leven • Onderliggende moleculaire mechanismen zijn grotendeels onbekend • De impact van prenatale fijnstofblootstelling op foetale transcriptoomprofielen is nog niet eerder bestudeerd	• Genexpressie van DNA-schade respons en olfactorische signaaltransductieketens werden gewijzigd door respectievelijk lang- en kortdurende prenatale fijnstofblootstelling bij zowel jongens als meisjes • Langdurige fijnstofblootstelling was geassocieerd met expressie van genen belangrijk voor de neurologische ontwikkeling (jongens) en expressie van defensine (meisjes). Kortdurende fijnstofblootstelling was geassocieerd met de mitochondriale functie (jongens) en de immuunrespons (meisjes)	• Dit is de eerste globale genexpressiestudie in navelstrengbloed om geslachtsafhankelijke pathways te identificeren die zijn veranderd door fijnstofblootstelling • De geïdentificeerde transcriptoom pathways kunnen nieuwe moleculaire inzichten verschaffen met betrekking tot het interactiepatroon van prenatale fijnstof-blootstelling met de biologische ontwikkeling van de foetus
Hoofdstuk 4	• Mitochondria zijn ○ energieproducerende organellen ○ de belangrijkste producenten en doelwitten van reactieve zuurstofsoorten (ROS)	• Transcriptoomanalyses hebben aangetoond dat fijnstofblootstelling geassocieerd was met mitochondriaal genoomonderhoud en apoptose voor kortdurende blootstelling, en met de elektronentransportketen (ETK) voor blootstelling op middellange termijn bij vrouwen	• De geïdentificeerde mitochondria-gelinkte genen en pathways impliceren een opregulatie van energie-producerende processen als een mogelijk mechanisme om voor fijnstof geïnduceerde mitochondriale schade te compenseren

Tabel 1. Samenvatting van dit proefschrift (vervolg)

Hoofdstuk	Achtergrondinfomatie	Wat deze studie bijbrengt	Conclusies en perspectieven
	• Fijnstof blootstelling is gelinkt aan gewijzigde ROS productie en mitochondriale functie • Zowel fijnstof blootstelling als mitochondriale schade zijn geassocieerd met verschillende chronische ziekten • Studies naar de impact van fijnstof blootstelling op mitochondria-gerelateerde genen zijn schaars	• Bij mannen was fijnstof blootstelling op middellange termijn geassocieerd met de citroenzuurcyclus • We hebben verschillende ETK genen kunnen valideren in een onafhankelijke studiepopulatie	

Hoofdstuk 5	• Telomeerlengte en mtDNA inhoud zijn
	  ○ gevoelig voor oxidatieve stress en ontsteking
	  ○ betrokken bij het ontstaan en de progressie van chronische ziekten
	• Het effect van overtollig lichaamsgewicht op telomeerlengte en mtDNA gehalte is onduidelijk
		• Telomeerlengte was negatief geassocieerd met de tailleomtrek bij volwassenen
		• De verandering in telomeerlengte over 4 jaar was negatief geassocieerd met verandering in de tailleomtrek
		• Een positieve trend tussen 4-jarige verandering in mtDNA en tailleomtrek bij mannen en een kromlijnige associatie bij vrouwen
			• Onze bevindingen suggereren dat het beperken van de gewichtstoename tijdens het ouder worden de natuurlijke telomeerverkorting kan vertragen en de veranderingen in mtDNA inhoud kan beïnvloeden, vooral in vrouwen
			• We adviseren toekomstige longitudinale studies om na te gaan of associaties verschillen tussen proefpersonen met toenemend en afnemend lichaamsgewicht over de opvolgingsperiode

Hoofdstuk 6	• MtDNA inhoud is een moleculaire biomerker van oxidatieve stress, ontsteking en mitochondriale schade
	• Verschillende studies hebben een verband aangetoond tussen mtDNA inhoud en fijnstof blootstelling
	  ○ Resultaten waren inconsistent met betrekking tot de richting van het effect
		• Langdurige fijnstof blootstelling was negatief geassocieerd
		  ○ met perifeer bloed mtDNA inhoud, vooral bij abdominaal obese deelnemers
		  ○ met verandering in perifeer bloed mtDNA inhoud over een opvolgingsperiode van ± 4 jaar, vooral in rokers en deelnemers met toenemende tailleomtrek gedurende de opvolgingsperiode
			• Zelfs onder de Europese richtlijnen voor luchtverontreiniging heeft fijnstof blootstelling een invloed op mtDNA inhoud
			• Toekomstige studies kunnen best rekening houden met mogelijke effect modificatie door lichaamsgewicht en potentiële synergie tussen roken en luchtvervuilingsblootstelling

Chapter 1

GENERAL INTRODUCTION

During the 20th century, considerable improvements in comfort and life expectancies shifted the primary public health problems from infectious to non-communicable (i.e. non-transmissible) chronic diseases including cancer, diabetes, chronic respiratory diseases, and cardiovascular diseases. Each year, 17 million people die prematurely as a result of the global chronic disease epidemic.[1] The invention of powered machines, vehicles, and electric appliances added to pleasure and life comfort, however, they also had unanticipated side effects. A sedentary lifestyle and increased calorie-dense food intake resulted in a tripling of obesity rates in men and a doubling in women since 1975.[2] In 2014, 39% of adults were overweight and 13% were obese.[3] High body-mass index and air pollution exposure were ranked, respectively, seventh and fifth in the top leading risk factors attributing to the global disease burden in 2016.[4] According to the World Health Organization, 80% of all heart diseases, strokes and diabetes type 2 and 40% of all cancers are linked to preventable risk factors.[1]

In order to ameliorate the continual increase in chronic diseases rates, understanding and defining the "exposome" and the link with chronic diseases is of utmost importance. As proposed by Dr. Wild in 2005, "the exposome encompasses life-course environmental exposures (including lifestyle factors), from the prenatal period onwards".[5] Here we focus on two environmental health treats: air pollution and obesity.

Exposures

Ambient air pollution

Since the Industrial Revolution, populations grew rapidly and emission of air pollution by factories, fossil-fuel based vehicles and homes increased. However, only after the occurrence of several acute episodes of lethal smog during the first half of the 20th century, the hazards of air pollution were recognized. In December 1930, a major air pollution disaster occurred in the Meuse Valley, Belgium. A temperature inversion just above the chimneys trapped particulate air pollutants which originated mainly from coal burning. The extreme exposure to particulate-smog made thousands of inhabitants ill and killed more than 60 people in five days. [6] Eighteen years later, a similar event took place in Donora, Pennsylvania, resulting in an estimated 20 deaths in five days.[7] Probably the most severe air

pollution episode happened in 1952 in London. The excess number of deaths, due to acute and persisting effects of the 1952 London smog, was estimated at 12,000 between December 1952 and February 1953.[8]

The awareness of the potential impact of air pollution exposure led to European regulatory guideline measures which remarkably improved air quality in most European countries over the past decades. Nonetheless, ambient air pollution is still one of the top risk factors for chronic disease burden.[9]

Air pollution characteristics

Air pollution can be divided into two categories: primary air pollutants, emitted directly from the source and secondary pollutants, formed by chemical interactions amongst primary pollutants. Primary pollutants can originate from natural (e.g. dust storms, forest fires, and volcanic eruptions) or anthropogenic sources (e.g. chemical processes and fuel combustion). Furthermore, we can classify air pollutants as gaseous or particulate pollutants.

A well-studied air pollutant in epidemiological studies is particulate matter 10 (PM_{10}), a complex mixture of small particulates and droplets, of organic and inorganic substances, with an aerodynamic diameter below 10 μm. Components are both primary and secondary pollutants and include metals (e.g. lead, cadmium and zinc), salts (e.g. sulfate, nitrates and sodium chloride), soil and dust particles, organic compounds (e.g. polycyclic aromatic hydrocarbons), biological compounds (e.g. allergens and endotoxins), and gasses (e.g. ammonia).[10-12]

The many emission sources of PM, which are hard to define, and the interplay between pollutants with each other and the environment makes that the composition and toxicity of particulate air pollution differs both spatially and temporally.[12, 13]

More recently, the focus of epidemiological studies has shifted from PM_{10} to $PM_{2.5}$. $PM_{2.5}$ is the fine fraction of PM_{10} with a diameter below 2.5 μm that can penetrate the peripheral regions of the bronchioles, and the ultrafine particles may cross the lung epithelium and enter the bloodstream. From there, they can be transferred to extrapulmonary target organs.[14] Moreover, ultrafine particles may be inhaled via the nose, enter the olfactory epithelium and can be further transported to the olfactory bulb, and they may even reach the brain.[15]

Health impacts of air pollution

Ambient air pollution is one of the leading environmental causes of the global disease burden. According to the European Environment Agency, in 2014, air pollution contributed to a total of 534,471 premature deaths in Europe, of which 11,181 in the Belgian population.[16]

Heart disease and stroke are responsible for 80% of the premature deaths attributed to air pollution, followed by lung disease and lung cancer.[17] PM was classified as carcinogenic in humans by the International Agency for Research on Cancer.[18]

It is recognized that the effects of air pollution in terms of human health depend on endogenous factors such as sex, age, and genetic factors and exogenous factors such as time spent outdoor, physical activity, and diet. Susceptible population groups are identified as those with pre-existing heart or lung disease, elderly, and children. Moreover, fetuses, which are indirectly exposed via the mother, are at greater risk due to the high rates of cell proliferation, organ development, the changing metabolic capabilities, the immature immune system, and decreased capacity of detoxification and DNA repair during critical fetal development stages.[19, 20] Recent reviews[21-27] suggested that air pollution is associated with adverse birth outcomes including low birth weight and preterm delivery. A causal association between air pollution exposure and adverse birth outcomes implies a large impact on overall public health given the widespread exposure to air pollution and the fact that birth outcomes are indicators of the health of newborns, infants and even have health implications later in life.[28] The hypothesis that adult diseases may have their roots in early life is referred to as the "Barker" or "Developmental Origins of Health and Disease" (DOHaD) hypothesis.[29, 30] Because of this well-known relation between pregnancy outcomes and infant mortality and health status in later life, the contribution of air pollutants to adverse birth outcomes is of great public health concern.

Air quality standards

The European ambient air quality directives report a European annual mean limit value of 40 $\mu g/m^3$ for PM_{10} and the daily mean PM_{10} concentration may not exceed 50 $\mu g/m^3$ more than 35 times in a year. For $PM_{2.5}$, the annual mean limit value is 25 $\mu g/m^3$. The guidelines of the WHO, which are not obligatory, are much stricter.

The annual limit value is 20 µg/m^3 for PM_{10} and 10 µg/m^3 for $PM_{2.5}$, and PM_{10} and $PM_{2.5}$ may not exceed respectively 50 µg/m^3 and 25 µg/m^3 more than 3 times a year.[16]

A European map of annual mean $PM_{2.5}$ concentrations in 2015 is given in Figure 1.[16]

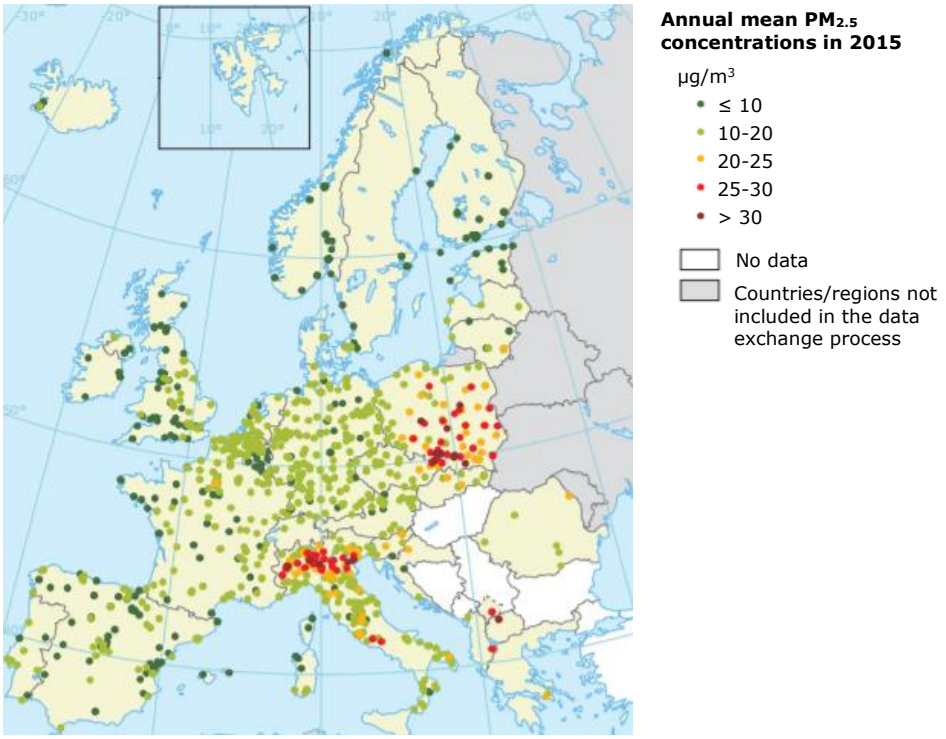


Figure 1. Annual mean $PM_{2.5}$ concentration in European countries, 2015. The red and dark red dots indicate stations reporting concentrations above the European limit value (25 µg/m^3). The dark green dots indicate stations reporting values below the WHO air quality guidelines (10 µg/m^3). *Reprinted from European Environmental Agency, Air quality in Europe - 2017 report.*[16]

Excess body weight

Besides ambient air pollution, excess body fat is a major risk factor in terms of human health in our modern society as it increases the risk of several non-communicable diseases.[31] Excess body weight results from an interplay between genetic, environmental, and behavioural factors.[32] The sedentary nature of office work, increased urbanization, the decreased role of active modes of transport, and the emergence of the computer as an online networking and gaming tool

resulted in considerable decreases in physical activity in adults and children. In combination with increased intake of calorie-dense foods, modern lifestyle resulted in an obesity epidemic in Europe.[33]

Health impacts of excess body weight

Some chronic diseases such as heart disease, type-2 diabetes, and certain cancers have been strongly linked with obesity.[31] Excess body weight is estimated to be the main contributor for 80% of all type 2 diabetes cases, 35% of ischaemic heart diseases, and 55% of hypertensive diseases among European adults.[34] Unfortunately, the rising trend in prevalence of obesity and obesity-related diseases in the last decades is expected to continue during the next 20 years.[35] By 2030, the prevalence of cancers, cardiovascular diseases, and diabetes has been predicted to reach an average of respectively 2,046, 4,672, and 3,990 cases per 100,000 across Europe. Lowering the body mass index by 5% would lower the prevalence of these diseases with respectively 2.1, 5.3 and 16.7% by 2030 across Europe.

Measures of obesity

Overweight and obesity are defined as a body mass index (BMI) greater or equal to 25 kg/m^2 and 30 kg/m^2, respectively. In 2014, 47.0% of adults (≥ 18 years old) in the European union (28 countries) were overweight and 15.4% were obese.[36] Corresponding numbers for Belgium were 48.8% and 13.7% respectively.

Whereas BMI is the official standard to diagnose overweight and obesity, it is a measure to define the degree rather than the distribution of body fat. Abdominal body fat deposition rather than the amount of total body fat is predictive for obesity-related comorbidities.[37, 38] Waist circumference, an index of abdominal body fat deposition, is increasingly applied to assess obesity-related risk complications. Abdominal obesity is defined as a waist circumference equal to or higher than 102 cm for men and equal to or higher than 88 cm for women.[39]

Molecular biomarkers

Biomarkers are referred to as measurable biological characteristics used to indicate some biological state of certain cells, tissues or individuals. In the area of

environmental health risk assessment, intermediate biomarkers of effect can be used to gain insight in the molecular mechanisms by which environmental exposures can alter human health. In recent years, growing interest has been dedicated to the role of mitochondrial DNA content and telomere length, both involved in cellular senescence, as potential mediators or biomarkers for chronic non-communicable diseases. Besides targeted approaches, the ongoing evolution in high-throughput omics technologies such as transcriptomics allows a genome-wide perspective on the molecular mechanisms underlying the association between environmental exposures and health effects.

Mitochondria

Mitochondria are the energy engines of cells and provide bioenergy in the form of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. They are involved in several fundamental metabolic and signalling processes including calcium signalling and programmed cell death. Each cell contains 100 to several 1000 mitochondria (Figure 2).[40, 41] The inner membrane of the mitochondria harbours the electron transport chains (ETC) which are responsible for the energy production in cells. Electrons, resulting from fatty acid and carbohydrates oxidation (tricarboxylic acid cycle), are transferred along the first four complexes (complex I-IV) of the ETC, to oxygen, generating water (Figure 2). This electron transfer results in a proton gradient, that is used by the ATP synthase to convert adenosine-5'-triphosphate (ADP) into ATP. As such, mitochondrial dysfunction can lead to energy deficits and eventually cellular dysfunction. Aside from a decreased mitochondrial bioenergetics efficiency, mitochondrial dysfunction can lead to increased premature electron leakage to oxygen, producing superoxide. This reactive oxygen species (ROS) is converted to less toxic components by antioxidant enzymes. In most cases this primary ROS is converted to hydrogen peroxide by an antioxidant called superoxide dismutase. In turn, hydrogen peroxide levels are maintained at low levels by glutathione peroxidase and catalase which convert hydrogen peroxide to water and oxygen. However, a disturbance in the balance between ROS generation and antioxidant defences due to mitochondrial dysfunctioning leads to elevated oxidative stress and has detrimental functional and structural consequences for cellular function.

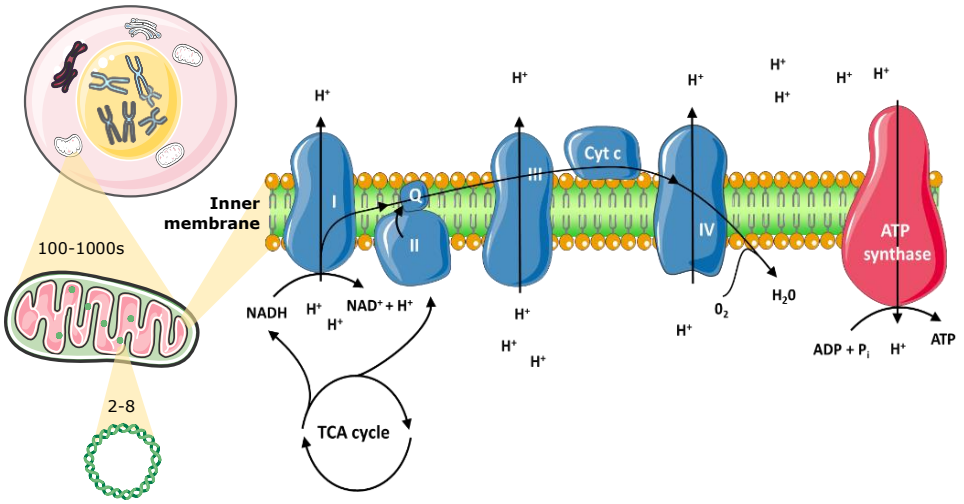


Figure 2. Schematic overview of the mitochondria, mitochondrial DNA and the electron transport chain. TCA: tricarboxylic acid; NAD: nicotinamide adenine dinucleotide; Cyt c: cytochrome c; ADP: adenosine diphosphate; ATP: adenosine triphosphate.

A unique property of mitochondria is that they contain their own DNA (mtDNA). Each mitochondrion harbours 2-8 copies of mtDNA.[40, 41] The human mtDNA is a circular, double-stranded molecule and spans only 16,569 base pairs. It contains 37 genes encoding 22 transfer RNAs, 2 ribosomal RNAs and 13 polypeptides of the ETC. However, the vast majority of the ETC proteins are encoded by nuclear DNA and a tight interplay between nuclear and mitochondrial gene products is necessary for mitochondrial maintenance. Other characteristics of mtDNA are the polymorphic nature, maternal inheritance pattern and the high mutation rate; 10- to 17-fold higher than the nuclear DNA.[42] The immediate proximity of the ROS generating ETC complexes, the lack of histones, and the limited repair mechanisms contribute to its susceptibility to DNA damage. Accumulation of mtDNA damage can cause disturbed mtDNA replication and elimination of damaged mtDNA, and as such lead to decreased levels of mtDNA.[43, 44] Alterations of mtDNA content are considered as a surrogate marker of mitochondrial functioning and damage.[45] As dysfunction of mitochondria is linked with several chronic age-related diseases such as diabetes, cancer, cardiovascular and neurodegenerative diseases,[45-48] they are an important research topic in the field of environmental health risk assessment.

Telomere length

Telomeres, also functioning as a "mitotic clock" or "replicometer", are DNA tandem repeats of the sequence TTAGGG that cap the ends of mammalian chromosomes and enhance chromosomal stability.[49] As a consequence of the end replication problem, telomeres become shorter every cell division until a critical stage is reached which eventually leads to replicative cell senescence or apoptosis (Figure 3).[50, 51] Telomeres can be re-elongated by an RNA-containing reverse transcriptase called telomerase.[52] Telomerase activity is necessary for survival of continuously dividing cells such as germ cells, stem cells, and most cancer cells[53, 54] which do not exhibit telomere shortening (Figure 3). However, telomerase is not active in most differentiated somatic cells. In leukocytes, telomere length decreases from approximately 10 kb at birth to around 8 kb at the age of 30 years.[55] The rate of telomere shortening is much higher in the first years of live than in adulthood, possibly reflecting the cellular proliferation rate[56, 57]

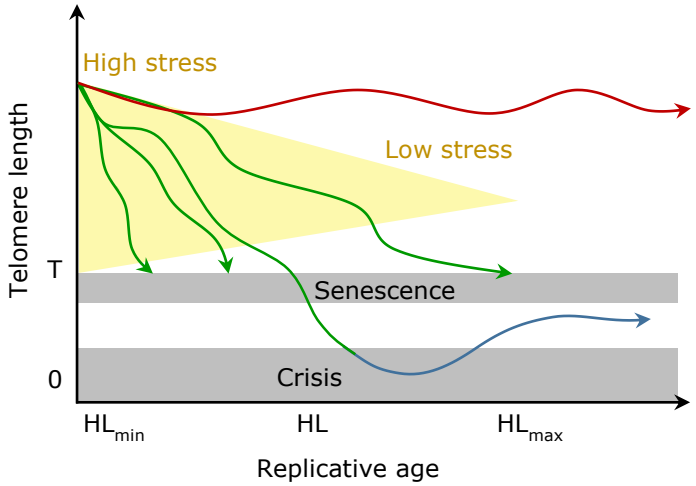


Figure 3. Schematic overview of telomere shorting in germ and stem cells (red) and in human somatic cells (green). If telomere length of somatic cells reaches a certain threshold (T), senescence is triggered. The number of replications until senescence is referred to as the Hayflick limit (HL). However, if the cell encompasses the senescence checkpoint, telomere shortening can continue leading to crisis. Somatic cells that can compensate for telomere loss by e.g. telomerase activation can become immortal (blue). The rate of telomere shortening (and consequently also the Hayflick limit) is affected by the cellular balance between oxidative stress and anti-oxidative defence as indicated by the yellow triangle. *Adjusted from Von Zglinicki T. Trends Biochem Sci 2002;27:339-44.*[58]

Besides telomere attrition by chronological aging, genetic background and cumulative exposure to oxidative stress induced by environmental and lifestyle factors, may accelerate telomere loss.[58-61] Telomeres, as guanine rich structures, are highly sensitive to oxidative modification.[62, 63] Furthermore, telomeric DNA is less efficient in the repair of single-strand breaks as compared to the bulk of the genome. The resulting high frequency of single-strand breaks in telomeric DNA interfere with the replication fork and as such cause telomere loss.[58] Besides, oxidative stress is observed to decrease telomerase activity in human leukemic cancer cells,[64] endothelial cells,[65, 66] and vascular smooth muscle cells[67]. Experimental studies showed that targeting antioxidants directly to the mitochondria or altering mitochondrial function and ROS production counteracted telomere loss and extended lifespan.[68, 69] Moreover, decreasing the generation of superoxide anion in the mitochondria by mild uncoupling, reduced telomere shortening.[70]

Accelerated telomere shortening is associated with the aetiology of many chronic age-related diseases including heart diseases,[71] stroke,[72] osteoporosis,[73] diabetes,[74] and certain cancers[75]. Telomere length may therefore contribute to the field of environmental epidemiology as an important biomarker of cumulative exposure to oxidative stress and a prognostic indicator of health complications later in life.

Transcriptomic profiles

The emergence of omics technologies offers opportunities to identify new biomarkers in the exposome context. Besides introducing novel candidate biomarkers of exposure and effect, the implementation of omics in the field of epidemiology may enhance mechanistic insight in chronic diseases and, via pathway discovery, it may provide a leap forward in the elucidation of exposure-induced adverse health effects.

To integrate omics technologies into life-course epidemiological health risk assessment the meet-in-the-middle approach is often used.[76] This approach gives insight into biological plausibility. In an exposome context, the goal is to identify intermediate biomarkers that are related to past environmental exposures and that are prognostic for adverse health effects later in life. Therefore, in a first step, the association between exposure and disease needs to be shown. Then, the

association of both the exposure and the adverse health effect with the intermediate omics biomarker needs to be assessed. In this doctoral dissertation the meet-in-the-middle approach is applied by combining previous study findings on the exposure-disease and biomarker-disease link with new evidence on the relation between exposure and possible intermediate omics biomarkers. We specifically assessed the transcriptome, which is defined as the complete set of gene transcripts in a cell or a tissue. By utilizing microarrays, one can quantitatively assess the responses of thousands of gene transcripts simultaneously.

A challenge in the field of transcriptomics, and other omics, is to extract biological insights from the generated high-throughput data. Pathway analyses may provide a plausible biological interpretation of the transcriptomic dataset and are especially useful in environmental observational studies where an exposure may give non-significant responses at the single-gene level.[77]

As blood sample collection is relatively non-invasive and feasible in an epidemiological setting, and the blood circulatory system serves as a connection between environmental exposure and many target tissues, blood cells have been suggested as a surrogate tissue to study molecular responses to environmental exposures.[78, 79]

Sex-differential effects

Previous studies have shown sex-specific responses in the field of environmental epidemiology. To further discuss potential underlying causes of sex disparities, it is necessary to distinguish between sex and gender. Sex refers to the biological component, defined via the chromosomal complement, and is reflected physiologically by the reproductive organs and sex hormones. Gender on the other hand comprises the behavioural, environmental, and social factors.

In the context of air pollution epidemiology, biological transport of environmental pollutants has been shown to be influenced by sex-differential factors including particle deposition,[80] alveolar blood-gas barrier permeability,[81] and airway hyperresponsiveness[82]. Besides, hormonal influences on immune, inflammatory, and oxidative stress responses may contribute to sex disparities in environmental related health effects.[83] Gendered explanations include work-related (co-

)exposure, smoking behavior, and exposure and response to physiosocial stressors that may all differ between women and men.[84] In most observational studies, PM is estimated at residential address or municipality which limits the disparities in the distribution of exposure estimates between women and men. However, the correlation between the estimated and the true PM exposure may differ by sex and as such may contribute to the sex-specific associations.

For fetuses and newborns, we assume that sex-specific effects are caused by biological sex differences including hormonal and chromosomal disparities.[85] Previously, Warembourg *et al.*[86] reported sex-specific associations between persistent organic pollutants and sex hormones in cord blood.

Regarding studies assessing obesity indices, sex-specific associations may be due to gender disparities in smoking behavior, dietary intake, alcohol consumption, physical activity and socio-behavioral factors. Biological explanations include differing fat storage patterns, fat mobilization and metabolism, and responses to both excess and insufficient fat stores.[87] In adult epidemiological studies, it is difficult to distinguish whether observed effect modification is due to sex or gender differences.

Main objectives

In this doctoral dissertation, we studied the effects of environmental stressors on biomarkers of chronic aging-related health effects including mtDNA content and telomere length, and defined transcriptomic profiles within a life-course epidemiology concept. We focused on ambient particulate matter exposure and excess body fat, two of the most harmful environmental risk factors and both linked with oxidative stress and inflammation. Because of feasibility, cost, and time issues in following individuals over their entire lifespan, we combined studies on data from birth and adult cohorts (Figure 4). The investigation of different life stages may elucidate critical windows during which interventions may be effective. As recommended by previous literature,[88, 89] we explored whether exposure-response associations were sex-specific.

The specific aims of this project were:

1. To investigate the effect of maternal PM_{10} exposure during pregnancy on birth weight and the risk of being small-for-gestational-age. We assessed effect modification by gestational age and potential non-linearity in the shape of the association (**chapter 2**).

2. To assess sex-specific transcriptomic responses to particulate air pollution exposure in newborns and in adults. First, we established newborn transcriptomic signatures of gestational short- and long-term exposure to $PM_{2.5}$ (**chapter 3**). Secondly, we performed transcriptome-wide analyses to study genes and pathways reflecting mitochondrial responses to PM_{10} exposure in an adult cohort (**chapter 4**). Thereupon, 13 mitochondria-associated genes were selected for further validation in an independent validation cohort. Within the validation study, we additionally investigated the association between PM exposure and mtDNA content.

3. To explore the sex-specific cross-sectional and longitudinal association of peripheral blood telomere length and mtDNA content with two obesity measures in a general adult population. BMI and waist circumference were considered as obesity measures. Besides performing cross-sectional analyses, we explored whether changes of obesity measures paralleled changes of telomere length and mtDNA content over a follow-up time of around 4 year. Smoothing plots were used to assess potential non-linearity in the exposure-response association (**chapter 5**).

4. To analyze the association between peripheral blood mtDNA content and long-term $PM_{2.5}$ exposure in a general adult population. We studied potential effect modification by sex, smoking status, and waist circumference (**chapter 6**).

Available data

Study populations

Population-based birth registry data

Birth registry data assessed in **chapter 2** were derived from two sources: the independent and regionally funded Study Centre for Perinatal Epidemiology (SPE)[90, 91] and the Belgian civil birth registration. Since 1986, the SPE collects data on births in all maternity units in Flanders and the University Hospital of Brussels. Flanders is the Dutch speaking Northern part of Belgium with an area of 13,522 km^2 and a population of about six million people. It has 68 fully equipped maternity units where 99.8% of all births occur. Besides, the SPE covers most of the home deliveries. For each newborn of at least 500 g (or a gestational age of at least 22 weeks when the birth weight is missing), an official perinatal form is completed by the midwife. The form is sent to the SPE, where data are subjected to an error detection program.[90] Information on parental education and national origin of the mother are recorded by the Belgian civil birth registration and linked to the medical birth certificates of the SPE. Because this linkage is only available from 1999 to 2009, we only considered births during this time period.

ENVIRONAGE (ENVIRonmental influence ON early AGEing)

Mother-child pairs are enrolled in the on-going ENVIR*ON*AGE birth cohort when they arrive at the East-Limburg Hospital in Genk (Belgium) for delivery.[92] The birth cohort was previously described in detail by Janssen and colleagues.[93] Briefly, participating mothers provide written informed consent for the collection of biospecimens, including placental biopsies and cord blood samples, as well as medical and lifestyle data. In the post-delivery ward the mothers complete study questionnaires to provide detailed information. On average, there are around 2000 live singleton births and 50 live twin births each year in the East-Limburg Hospital. The overall participation rate of the ENVIR*ON*AGE birth cohort is 61%. In **chapter 3**, we considered a subset of 142 newborns, for which whole genome mRNA expression was measured, born between Friday 1200 hours and Monday 0700 hours from March 20th 2010 until March 9th 2014.

FLEHS (Flemish Environment and Health Study)

Until now, three FLEHS campaigns were carried out (FLEHS I: 2002-2006, FLEHS II: 2007-2011, FLEHS III: 2012-2015) and included in total 5825 participants which provided informed consent.[94] For each campaign, different age groups were recruited: mother-newborn pairs, adolescents (10-15 years old), adults between 20 and 40 years old, and adults between 50 and 65 years old. The latter were only considered in FLEHS I and FLEHS III.

In **chapter 4**, we selected a subset of 98 adults (50-65 years old) of FLEHS I for whole genome microarray analysis and 169 adults (50-65 years old) of FLEHS III for validation. Demographic data, lifestyle factors and health parameters were provided through an extensive self-assessment questionnaire and blood samples were collected by nurses. All participants were able to fill in Dutch questionnaires and lived in the Flemish study area for five years (FLEHS I) or in Flanders for at least 10 years (FLEHS III).

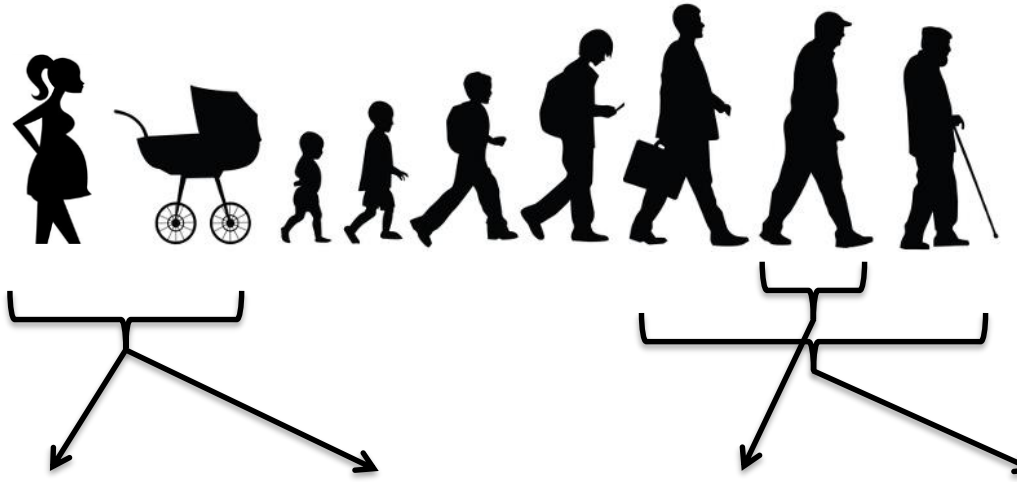
FLEMENGHO (Flemish Study on Environment, Genes, and Health Outcomes)

From 1985 to 2004, FLEMENGHO recruited 3360 participants living in a geographically defined area in the Northern part of Belgium. The recruitment procedure was previously described in detail.[95] The participants provided written informed consent and were repeatedly followed-up. Clinical and biochemical measures, and extensive study questionnaires were collected using the same standardized methods in all examination phases. For **chapters 5 and 6**, we considered participants aged between 18 and 90 years old, for which blood samples were available and collected between 2005 and 2013. For 228 adults, peripheral blood mtDNA content and telomere length were measured at two examination days with around 4 years in between.

Exposure data

Average daily PM_{10} and $PM_{2.5}$ concentrations ($\mu g/m^3$) were obtained from the Belgian Interregional Environment Agency (IRCEL) and modelled at the level of the municipality of residence (**chapter 2**) or for each participants' residential address (**chapters 3, 4, and 6**) using a spatial temporal interpolation method

(Kriging)[96] (**chapter 2**). In combination with a dispersion model (IFDM)[97-99] that uses emissions from line sources and point sources, this model chain provides PM values in a dense irregular receptor grid (maximum grid cell size of 25x25m) (**chapters 3, 4, and 6**). It is expected that $PM_{2.5}$ has stronger adverse health effects than the coarser part of PM_{10}. Therefore in **chapter 3 and 6**, $PM_{2.5}$ exposure was chosen as the variable of interest. However, since $PM_{2.5}$ data are only available from 2005 onwards and PM_{10} data already from 1997, we used PM_{10} data in **chapter 2 and 4**.



Birth registry data	ENVIR*ON*AGE	FLEHS	FLEMENGHO

• Chapter 2
• 1999-2009
• Exposure of interest
  ○ Prenatal PM_{10}
• Birth outcomes:
  ○ Birth weight
  ○ SGA
	• Chapter 3
	• 2010-2014
	• Exposure of interest
	  ○ Prenatal $PM_{2.5}$
	• Outcome:
	  ○ Transcriptomics
		• Chapter 4
		• 2004-2005 (FLEHS I), 2014 (FLEHS III)
		• Exposure of interest
		  ○ PM_{10}
		• Outcomes:
		  ○ Transcriptomics
		  ○ MtDNA content
			• Chapter 5 and 6
			• 2010-2014
			• Follow-up study
			• ± 4 years in between
			• Exposures of interest
			  ○ Obesity measures
			  ○ $PM_{2.5}$
			• Outcomes:
			  ○ MtDNA content
			  ○ Telomere length

Figure 4. Overview per chapter

Chapter 2

FETAL GROWTH AND MATERNAL EXPOSURE TO PARTICULATE AIR POLLUTION – *MORE MARKED EFFECTS AT LOWER EXPOSURE AND MODIFICATION BY GESTATIONAL DURATION*

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Environmental Research 2015; 140:611–8

Abstract

Background: While there is growing evidence that air pollution reduces fetal growth, results are inconclusive with respect to the gestational window of effect. We investigated maternal exposure to particulate matter (PM_{10}) in association with birth weight and fetus growth with a focus on the shape of the association and gestational age at birth as a potential effect modifier.

Methods: The study population consisted of 525,635 singleton live births in Flanders (Belgium) between 1999 and 2009. PM_{10} exposure at maternal residence was averaged over various time windows. We used robust linear and logistic regression to estimate the effect of PM_{10} on birth weight and small-for-gestational-age (SGA). Segmented regression models were applied for non-linear associations.

Results: Among moderately preterm (32-36 weeks) and term (>36 weeks) births, we found significant lower birth weight with increased exposure for all studied time windows. The estimated reduction in birth weight for a 10 $\mu g/m^3$ increase in average PM_{10} during pregnancy was 39.0 g (95% CI 26.4 to 51.5) for moderately preterm births and 24.0 g (95% CI 20.9 to 27.2) for term births. The corresponding odds ratios for SGA were 1.19 (95% CI 1.07 to 1.32) and 1.09 (95% CI 1.06 to 1.12) respectively. Segmented regression models showed stronger effects of PM_{10} on fetal growth at lower concentrations.

Conclusions: Maternal PM_{10} exposure was significantly associated with a reduction in fetal growth among term and moderately preterm births, with a tendency of stronger effects for the latter and a flattening out of the slope at higher PM_{10} concentrations.

Introduction

The effects of air pollution exposure on the development of the fetus have become an area of increasing focus. Fetal growth is an important indicator of developmental problems and reduced growth is associated with diseases in adulthood including an elevated risk for (1) cardiovascular problems such as coronary heart disease and stroke,[100] (2) hypertension,[101] (3) type 2 diabetes,[102] and (4) mental issues resulting in an increased trend of prescriptions for antipsychotics, antidepressants, and hypnotics/sedatives in young adulthood.[103]

Several studies investigated whether maternal air pollution exposure is associated with adverse birth outcomes, such as low birth weight (LBW, <2,500 g), small-for-gestational-age (SGA), premature birth (<37 weeks of gestation), birth defects, and stillbirth.[22, 24, 25] Most reviews concluded that there is growing evidence for an association between prenatal exposure to air pollution and fetal growth.[22, 24, 25, 104, 105] Meta-analyses showed substantial heterogeneity between studies that may result from differences in outcome definitions, air pollutants considered, quantification of exposure, exposure windows, study populations and regions, statistical methods, and (inadequate) adjustment for confounders.[106-108] One of the recommendations is that the variation in effects by exposure window should be further explored. The interpretation of air pollution effects on birth weight is further complicated by the fact that they can reflect an influence on length of gestation, on fetal growth, or both. To capture only the latter, most studies have restricted their study population to term births. Moreover, the majority of studies assumed that the effect of air pollution on fetal growth is linear, whereas a few studies have suggested that this may not be the case.[109, 110]

Here, we investigated the association between fetal growth and maternal PM_{10} exposure during different time windows of pregnancy, including some critical exposure windows around the start and end of pregnancy. Relatively novel aspects of this study are the investigation of the shape of the association and the assessment of potential effect modification by gestational age. For the latter, we stratified the analysis by gestational age group (<32, 32-36, >36 weeks).

Methods

Data

The Study Centre for Perinatal Epidemiology (SPE) obtained information on all births in Flanders. Flanders is the Dutch speaking Northern part of Belgium with an area of 13,522 km^2 and a population of about six million people. It has 68 maternity units where 99.8% of all births (≥500 g) occur. For each newborn of at least 500 g, an official perinatal form is completed, mostly by the midwife, which contains information on birth weight and gestational age. The form is sent to the SPE, where an error detection program controls all data and feedback is provided.[90] The qualitative assessment of data obtained by the SPE showed that there is less than 5% difference between electronic data and data derived from medical files.[90] Gestational age is counted as the number of weeks starting from the first day of the last menstrual period and is corrected based on the measured crown-rump length from the first ultrasound. Information on national origin of the mother and education are gathered through linkage of the medical birth certificates of the SPE with official birth declarations. Because this linkage is only available from 1999 to 2009, we only considered births during this time period. We restricted our study population to live-born singleton births delivered at 24-44 weeks of gestation. Outcomes of interest in this study were birth weight and SGA. Neonates were classified as SGA when the birth weight was below the 10^{th} percentile of the birth weight for a given gestational age and gender of the newborn in the study population.

Average daily PM_{10} concentrations were obtained from the Belgian Interregional Environment Agency (IRCEL). In the region of Flanders, nineteen monitoring stations, situated on average 25 km apart from each other, have been in use since 1998. Daily levels of air pollution are interpolated by means of a land use regression model (RIO), described by Janssen and colleagues.[111] This provides interpolated air pollution estimates on a 4 x 4 km^2 grid. Since home address information was not available due to privacy laws in Belgium, population-weighted averages were calculated per municipality. Meteorological data consisting of mean daily air temperature and relative humidity, measured at the central and representative station of Uccle (Brussels, Belgium), were provided by the Belgian Royal Meteorological Institute. Apparent temperature, an index of human

discomfort incorporating relative humidity, was computed by using a standard formula.[112, 113]

Maternal PM_{10} exposure and mean apparent temperature were averaged over different time windows: the entire pregnancy, the two weeks around conception (one week before until six days after conception), the two weeks after conception, each of the three trimesters, and the last month of pregnancy. Average exposure during the third trimester was only calculated for moderately preterm and term births, because this time window is very short or non-existent for extremely preterm births (<32 weeks). The research protocol was approved by the medical ethics committee of the Hasselt University.

Statistical analyses

To study the association between fetal growth and PM_{10}, we applied robust linear regression models for the continuous outcome birth weight and logistic models for the binary outcome SGA. Since we expected the effects of PM_{10} exposure and covariates on birth outcomes to depend on gestational age, analyses were performed within the following three groups: extremely preterm (<32 weeks), moderately preterm (32-36 weeks), and term (>36 weeks) births.

The shape of the association between birth outcomes and maternal PM_{10} exposure was explored by the use of smoothing plots (natural cubic splines with 4 degrees of freedom). Because different exposure windows showed a breakpoint in the dose-response curve with relatively linear effects before and after that point, we additionally performed segmented (piecewise linear) regression analyses.

The choice of covariates adjusted for in the analysis was based on previous study findings on this topic.[114-116] Models included indicator variables for year of birth, season of conception (winter, spring, summer, autumn), parity (first, second, higher-order birth), maternal age group (<25, 25-34, >34 years), marital status (married, unmarried), maternal and paternal education (lower secondary or less, higher secondary, higher education), national origin of the mother (Europe, Asia, Middle-East, Africa, North-America, South-America, Oceania), province of residence (West Flanders, East Flanders, Antwerp, Flemish Brabant, Limburg), and a linear term for apparent temperature. For the regression models fitting birth

weight, we additionally adjusted for infant's sex and gestational age (linear and quadratic term).

Population attributable fractions (PAFs) were calculated as the proportion of SGA births that could be avoided if average maternal PM_{10} exposure during pregnancy was below 20 µg/m^3, which is the WHO annual guideline value.[117] PAFs are calculated with the formula $PAF = \sum (OR_{c-20} - 1)/OR_{c-20}$.[118, 119] OR_{c-20} is the odds ratio of SGA comparing a maternal exposure concentration *c* with an exposure of 20 µg/m^3.

In a secondary analysis, we examined whether specific subgroups were more vulnerable to the effects of maternal PM_{10} exposure. We stratified by gender of the newborn, parity, maternal age group, maternal and paternal educational level, and season of conception. Because of the small number of extremely and moderately preterm births within subpopulations, these secondary analyses were only performed in the term group.

A total of 122,936 births (19%) was not included in the final study population because of missing values for at least one of the covariates in the model (2,399 missing values for marital status, 41,847 for maternal education, 69,492 for paternal education and 55,492 for maternal origin). In a sensitivity analyses, models without adjustment for aforementioned variables (one model for the total population and one for the final study population) were used to examine the impact of the exclusion of these births.

Estimates are reported for a 10 µg/m^3 increment in PM_{10} exposure. All analyses were performed by using SAS version 9.2 (SAS Institute, Cary, North Carolina, USA). A *P*-value less than 0.05 was considered significant.

Results

There were 672,261 live births in Flanders between 1999 and 2009. From these, 23,551 (3.5%) non-singleton births and a further 139 (0.02%) births with a gestational age below 24 or above 44 weeks were excluded. After excluding 122,936 (19.0%) deliveries with missing information on one of the covariates, the final study population consisted of 525,635 births. Mean birth weight was 3,350 g and a total of 49,605 (9.4%) of newborns were SGA (Table 1). Of the 30,982

(5.9%) preterm births, 27,912 (90.1%) were moderately preterm and 3,070 (9.9%) were extremely preterm. Most neonates were firstborn (46.6%) from mothers between 25 and 34 years old (72.7%). Mean birth weight was lowest (and the percentage of SGA babies was highest) for girls, firstborns, young mothers, unmarried mothers, and mothers and fathers with low education.

Table 1. Descriptive statistics of the study population (n=525,635), Flanders, 1999-2009. Values are percentages or means (10-90th percentiles)

Characteristic	Value	% of all births	Mean birth weight [g] (10-90th percentiles)	% SGA
Total		100.0	3,350 (2,750-3,960)	9.4
Gestational age	<32 weeks	0.6	1,259 (745-1,795)	9.6
	32-36 weeks	5.3	2,592 (1,940-3,200)	9.5
	>36 weeks	94.1	3,399 (2,850-3,980)	9.4
Gender	Boy	51.3	3,413 (2,800-4,030)	9.4
	Girl	48.7	3,284 (2,700-3,880)	9.5
Parity	1	46.6	3,279 (2,680-3,885)	11.9
	2	35.4	3,408 (2,825-4,000)	7.2
	≥ 3	18.0	3,419 (2,795-4,060)	7.3
Maternal age	<25 years	15.3	3,260 (2,660-3,865)	12.5
	25-34 years	72.7	3,366 (2,770-3,970)	8.8
	>34 years	12.1	3,371 (2,725-4,020)	9.5
Marital status	Unmarried	31.1	3,296 (2,680-3,915)	11.5
	Married	68.9	3,375 (2,780-3,980)	8.5
Maternal education	Low	12.6	3,282 (2,650-3,925)	12.6
	Medium	40.7	3,310 (2,700-3,930)	10.7
	High	46.7	3,405 (2,820-4,000)	7.4
Paternal education	Low	14.7	3,286 (2,660-3,920)	12.4
	Medium	46.9	3,322 (2,710-3,940)	10.3
	High	38.3	3,409 (2,830-4,000)	7.3
Maternal origin	Europe	89.9	3,349 (2,750-3,960)	9.4
	Asia	1.1	3,274 (2,680-3,890)	11.9
	Middle East	3.6	3,321 (2,745-3,920)	10.5
	Africa	4.9	3,410 (2,810-4,038)	8.5
	North-America	0.1	3,449 (2,900-4,050)	5.0
	South-America	0.4	3,337 (2,785-3,916)	8.8
	Oceania	0.0	3,304 (2,710-3,880)	10.2
Season of conception	Winter	23.5	3,346 (2,740-3,960)	9.6
	Spring	25.0	3,344 (2,740-3,950)	9.7
	Summer	25.4	3,356 (2,760-3,970)	9.2
	Autumn	26.2	3,354 (2,750-3,970)	9.3

SGA: Small-for-gestational-age

Table 2 provides the distribution of average PM_{10} exposures during the different time windows. Average PM_{10} was close to 31 µg/m^3 for all windows. Trimester exposures were highly correlated with entire pregnancy exposure (linear correlation coefficient (r) >0.8) and moderately correlated with each other (r between 0.5 and 0.7) (Table 3). Correlations between time windows around conception and late pregnancy periods were low (r<0.4).

Table 2. Distribution of PM_{10} [µg/m^3] exposure in different time windows during pregnancy, Flanders, 1999-2009

			Percentiles			
Exposure window	**Mean**	**(SD)**	**5th**	**25th**	**75th**	**95th**
Entire pregnancy	31.24	(-5.88)	22.42	26.76	35.53	41.46
Two wks around conception	31.52	(-9.60)	18.33	24.84	36.71	49.37
Two wks after conception	31.55	(-9.62)	18.34	24.87	36.74	49.40
First trimester	31.46	(-6.54)	21.56	26.73	35.84	42.78
Second trimester	31.25	(-6.70)	21.03	26.42	35.73	42.84
Third trimester	30.96	(-7.07)	20.09	25.86	35.60	43.32
Last month	30.87	(-8.18)	18.73	25.00	35.92	45.51

SD: Standard deviation

Table 3. Correlation coefficients between average PM_{10} exposure in different time windows during pregnancy, Flanders, 1999-2009*

Exposure window	Entire pregnancy	Two wks around conception	Two wks after conception	First trimester	Second trimester	Third trimester	Last month
Entire pregnancy	1						
Two wks around conception	0.53	1					
Two wks after conception	0.56	0.75	1				
First trimester	0.86	0.58	0.65	1			
Second trimester	0.90	0.42	0.42	0.67	1		
Third trimester	0.84	0.39	0.38	0.56	0.67	1	
Last month	0.72	0.35	0.34	0.49	0.55	0.86	1

*P<0.001 for all correlations

Among moderately preterm and term births, we observed significant negative associations between PM_{10} and birth weight for all studied time windows, whereas we did not find any significant association among extremely preterm births (Table 4). Effects of PM_{10} on birth weight were always stronger for moderately preterm than for term births and were highest for entire pregnancy exposure in both groups: for a 10 µg/m^3 increase in average PM_{10} during pregnancy, birth weight

decreased by 39.0 g (95% CI 26.4 to 51.5) among moderately preterm births and by 24.0 g (95% CI 20.9 to 27.2) among term births.

Smoothing plots of the association between PM_{10} and change in birth weight suggested the existence of a breakpoint in the shape of the association, with relatively linear slopes before and after the breakpoint. Slopes were steepest at lower levels of PM_{10} (below approximately 35 $\mu g/m^3$) and flattened out at higher levels. The shape of the association is shown for entire pregnancy (Figure 1A) and first trimester (Figure 1B) PM_{10} exposure among term births. Supplementary Figures S1 and S2 display the exposure-response curves for the other pregnancy windows and gestational age groups. The decrease in slope above the breakpoint was significant for all time windows among term births, and for the time windows two weeks around conception and second trimester among moderately preterm births. Estimates for the significant breakpoints varied from 29.8 to 40.5 $\mu g/m^3$.

Table 4. Association between birth weight and maternal exposure to PM_{10} in different time windows during pregnancy

	Linear regression	Segmented linear regression			
	Change in weight [g]	Breakpoint [µg/m³]	*P*-value†	Change in weight [g] below breakpoint	Change in weight [g] above breakpoint
>36 weeks (n=494,653)					
Entire pregnancy	-24.0 (-27.2 to -20.9)*	35.4 (31.9 to 38.9)	<0.0001	-28.3 (-32.3 to -24.3)*	-14.2 (-20.7 to -7.6)*
Two wks around conception	-4.0 (-5.5 to -2.8)*	29.8 (24.4 to 35.3)	<0.0001	-10.9 (-13.0 to -6.0)*	-1.9 (-3.8 to -0.1)*
Two wks after conception	-5.0 (-7.0 to -3.6)*	33.9 (28.6 to 39.2)	<0.0001	-8.7 (-11.4 to -6.1)*	-2.0 (-4.2 to 0.2)
First trimester	-14.8 (-17.4 to -12.3)*	40.5 (37.9 to 43.1)	<0.0001	-17.4 (-20.3 to -14.5)*	5.7 (-3.7 to 15.0)
Second trimester	-16.6 (-19.1 to -14.0)*	31.2 (23.4 to 38.9)	0.041	-20.6 (-25.0 to -16.1)*	-14.2 (-17.7 to -10.7)*
Third trimester	-14.8 (-17.2 to -12.4)*	36.2 (31.7 to 40.7)	<0.0001	-18.1 (-21.3 to -15.0)*	-6.8 (-11.8 to -1.7)*
Last month	-8.8 (-10.6 to -7.0)*	31.9 (24.1 to 39.7)	0.013	-12.1 (-15.5 to -8.8)*	-5.9 (-8.7 to -3.2)*
32-36 weeks (n=27,912)					
Entire pregnancy	-39.0 (-51.5 to -26.4)*	32.6 (24.1 to 41.1)	0.071	N/A	N/A
Two wks around conception	-13.9 (-19.5 to -8.2)*	31.5 (23.0 to 40.0)	0.007	-29.1 (-41.5 to -16.6)*	-5.4 (-13.6 to 2.8)
Two wks after conception	-11.7 (-17.3 to -6.1)*	29.4 (18.7 to 40.1)	0.523	N/A	N/A
First trimester	-29.0 (-39.4 to -18.6)*	29.9 (15.7 to 44.0)	0.766	N/A	N/A
Second trimester	-26.0 (-36.5 to -15.5)*	33.7 (26.9 to 40.4)	0.033	-38.0 (-53.3 to -22.6)*	-10.5 (-27.9 to 7.0)
Third trimester	-16.5 (-25.0 to -7.9)*	30.6 (2.2 to 59.0)	0.690	N/A	N/A
Last month	-10.2 (-17.7 to -2.7)*	14.6 (11.4 to 17.8)	0.344	N/A	N/A
<32 weeks (n=3,070)					
Entire pregnancy	6.5 (-18.8 to 31.8)	30.6 (25.5 to 35.8)	0.110	N/A	N/A
Two wks around conception	0.5 (-12.6 to 12.4)	44.6 (14.0 to 75.1)	0.804	N/A	N/A
Two wks after conception	-5.5 (-17.4 to 6.5)	30.6 (20.7 to 40.5)	0.151	N/A	N/A
First trimester	9.6 (-11.6 to 30.8)	26.6 (22.9 to 30.3)	0.060	N/A	N/A
Second trimester	-1.8 (-23.9 to 20.1)	43.1 (24.7 to 61.4)	0.620	N/A	N/A
Last month	5.1 (-10.2 to 20.4)	30.3 (23.6 to 37.0)	0.078	N/A	N/A

Estimates (95% CI) are expressed as the change in birth weight for a 10 µg/m³ increase in PM_{10} and are adjusted for year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence, apparent temperature, infant's sex and gestational age.

*P<0.05

†Significance of the change in slope above the breakpoint; N/A = Not applicable (slope change not significant)

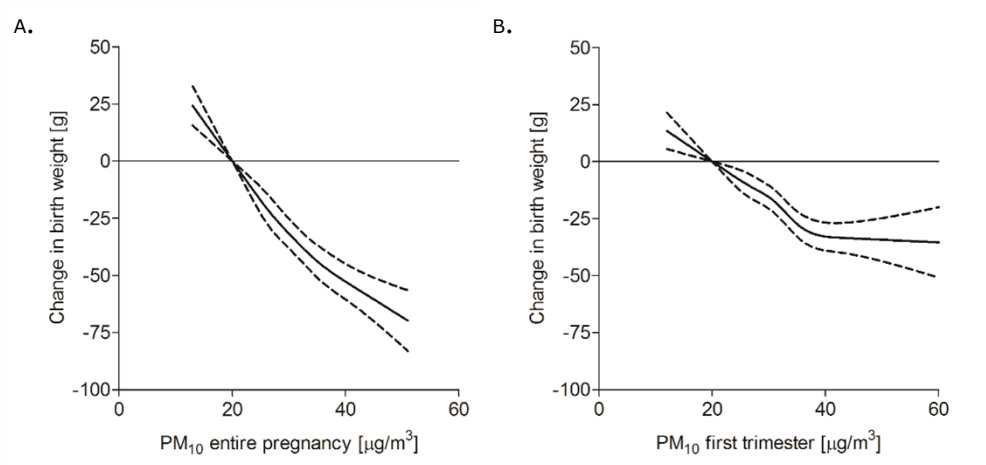

Figure 1. Shape of the association between term birth weight and maternal PM_{10} exposure during the entire pregnancy (A) and during the first trimester (B). Estimates (solid line) and 95% CI (dashed lines) represent the change in birth weight relative to the reference value of 20 µg/m^3. PM_{10} exposure was modeled using a natural cubic spline with 4 degrees of freedom and estimates are adjusted for year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence, apparent temperature, infant's sex, and gestational age.

Similar results were found when considering SGA as an outcome (Table 5). For moderately preterm and term births, the association between SGA and maternal PM_{10} exposure was mostly significant (except for some time windows at the end of pregnancy), but no significant associations were found among extremely preterm births. Odds ratios for a 10 µg/m^3 increase in PM_{10} were highest for the entire pregnancy time window: 1.19 (95% CI 1.07 to 1.32) for moderately preterm births and 1.09 (95% CI 1.06 to 1.12) for term newborns. The population attributable fraction for a decrease in average PM_{10} exposure during pregnancy to 20 µg/m^3 was 24% (95% CI 10 to 35) for moderately preterm SGA newborns and 13% (95% CI 9 to 16) for term SGA newborns. The decrease in slope after the breakpoint was significant for the time windows entire pregnancy, two weeks after conception, and second trimester among term births, and the time windows entire pregnancy and last month among moderately preterm births. Estimates for the significant breakpoints ranged from 27.2 to 40.0 µg/m^3.

Table 5. Association between SGA and maternal exposure to PM_{10} in different time windows during pregnancy

	Logistic regression	Segmented logistic regression			
	Odds ratio SGA	Breakpoint [µg/m³]	*P*-value†	Odds ratio SGA below breakpoint	Odds ratio SGA above breakpoint
>36 weeks (n=494,653)					
Entire pregnancy	1.09 (1.06 to 1.12)*	27.2 (23.5 to 31.0)	0.017	1.19 (1.10 to 1.28)*	1.07 (1.04 to 1.11)*
Two wks around conception	1.01 (1.00 to 1.02)*	30.0 (26.8 to 33.3)	0.098	N/A	N/A
Two wks after conception	1.02 (1.01 to 1.03)*	31.5 (27.9 to 35.1)	0.049	1.04 (1.02 to 1.07)*	1.01 (0.99 to 1.02)
First trimester	1.06 (1.04 to 1.08)*	35.5 (24.4 to 46.5)	0.701	N/A	N/A
Second trimester	1.07 (1.05 to 1.10)*	29.1 (24.8 to 33.3)	0.007	1.13 (1.08 to 1.19)*	1.05 (1.02 to 1.08)*
Third trimester	1.04 (1.02 to 1.06)*	26.9 (22.0 to 31.8)	0.193	N/A	N/A
Last month	1.02 (1.00 to 1.03)*	31.5 (25.5 to 37.5)	0.382	N/A	N/A
32-36 weeks (n=27,912)					
Entire pregnancy	1.19 (1.07 to 1.32)*	34.5 (24.5 to 44.5)	0.028	1.33 (1.14 to 1.53)*	0.98 (0.79 to 1.20)
Two wks around conception	1.09 (1.04 to 1.15)*	32.2 (16.2 to 48.2)	0.447	N/A	N/A
Two wks after conception	1.07 (1.02 to 1.12)*	14.3 (11.4 to 17.1)	0.453	N/A	N/A
First trimester	1.16 (1.06 to 1.27)*	54.4 (48.1 to 60.7)	0.248	N/A	N/A
Second trimester	1.13 (1.03 to 1.24)*	51.5 (37.5 to 65.6)	0.343	N/A	N/A
Third trimester	1.04 (0.97 to 1.12)	17.8 (8.4 to 27.2)	0.215	N/A	N/A
Last month	1.01 (0.94 to 1.08)	40.0 (32.1 to 47.9)	0.004	1.09 (1.00 to 1.18)*	0.77 (0.64 to 0.94)*
<32 weeks (n=3,070)					
Entire pregnancy	0.96 (0.70 to 1.34)	23.3 (17.4 to 29.2)	0.054	N/A	N/A
Two wks around conception	1.04 (0.89 to 1.21)	14.9 (14.0 to 15.7)	0.665	N/A	N/A
Two wks after conception	1.12 (0.97 to 1.31)	68.2 (26.7 to 109.6)	0.697	N/A	N/A
First trimester	0.98 (0.74 to 1.29)	26.1 (20.3 to 31.8)	0.069	N/A	N/A
Second trimester	0.94 (0.70 to 1.24)	17.0 (16.6 to 17.3)	0.907	N/A	N/A
Last month	0.96 (0.79 to 1.17)	31.7 (19.6 to 43.9)	0.101	N/A	N/A

Estimates (95% CI) are expressed as odds ratios for the risk of small-for-gestational-age (SGA) for a 10 µg/m³ increase in PM_{10} and are adjusted for year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence and apparent temperature.

*P<0.05

†Significance of the change in slope above the breakpoint; N/A = Not applicable (slope change not significant)

Secondary analyses indicated that the association between birth weight and maternal exposure to air pollution was stronger for increasing number of previous births and weaker for increasing maternal age and parental education (Figure 2A). The higher susceptibility of younger mothers was also observed for SGA (Figure 2B).

Models without adjustment for marital status, maternal and paternal education, and national origin of the mother (supplementary Table S1) suggested that estimates were fairly robust to the exclusion of subjects with missing covariate information. In these models estimates of weight decrease were slightly lower for the final study population than for the total study population among term births, and vice versa among moderately preterm births. A comparison with results from the main analysis showed that estimates of weight decrease were generally lowest in the main analyses, except for some exposure windows among moderately preterm births.

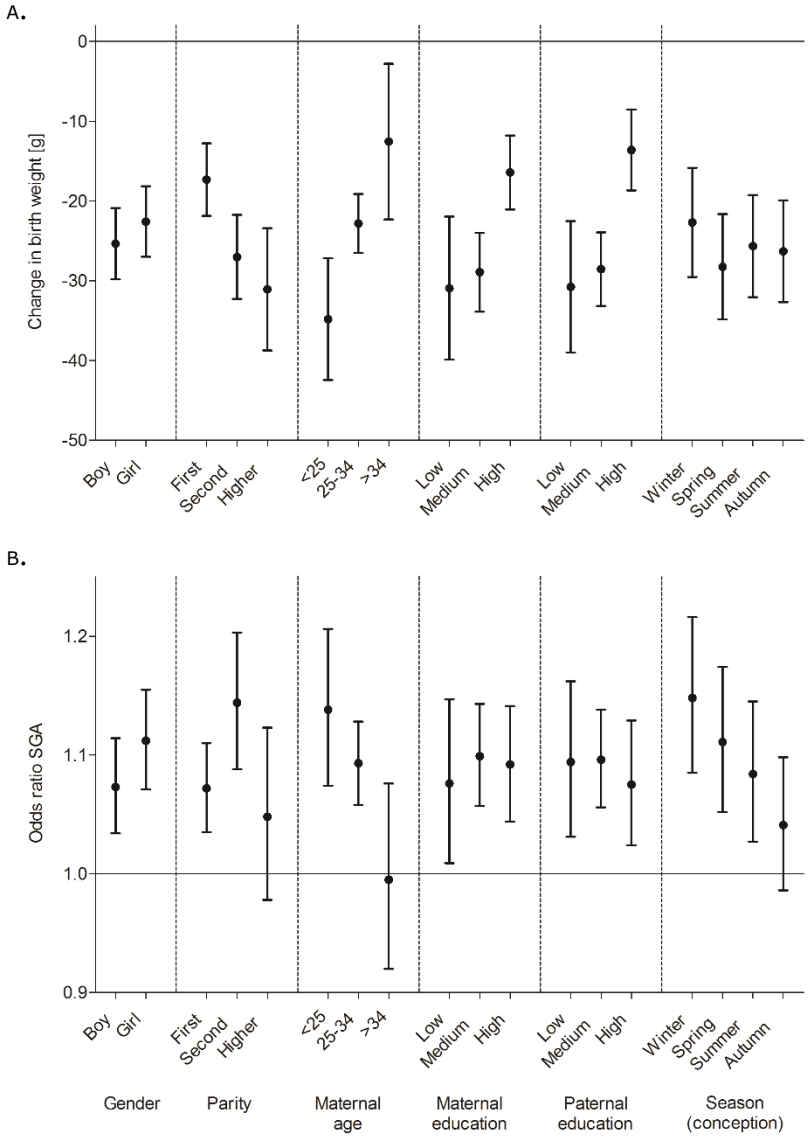

Figure 2. Association of term birth weight (A) and small-for-gestational-age (SGA) (B) with average PM_{10} exposure during pregnancy for different subpopulations. Estimates (point) and 95% CI (error bars) represent the changes in birth weight (A) and odds ratios for SGA (B) for a 10 $\mu g/m^3$ increase in maternal PM_{10} exposure and were adjusted for year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence, and apparent temperature and for birth weight (A) additionally for infant's sex, and gestational age.

Discussion

For infants born after 31 weeks of gestation, we observed significant associations between *in utero* PM_{10} exposure and birth weight as well as SGA. The effects of maternal PM_{10} exposure on both outcomes were considerably higher for moderately preterm neonates (32-36 weeks) than for those born at term. Susceptibility to air pollution was found to be higher for multiparous women and for lower maternal age and parental education. For both birth weight and SGA, the estimated breakpoint and the significance of the change in slope depended on the studied time window. In general, significant breakpoints were estimated to lie around 35 $\mu g/m^3$ and the estimated decrease in birth weight was largest for PM_{10} concentrations below this level. Twenty seven percent of our study population had an average pregnancy exposure above 35 $\mu g/m^3$.

Effect modification by gestational age was also observed in the association between second-trimester traffic-related air pollution (nitrogen dioxide, NO_2) and fetal growth restriction with a stronger effect among women subsequently delivering preterm.[120] However, these authors did not examine potential differences in susceptibility between early and late preterm babies. We did not observe a significant association between air pollution exposure and fetal growth for extremely preterm births. Since most of the fetal growth occurs in the third trimester, it might be that extremely preterm babies were born too early to see the effect of air pollution on growth. On the other hand, term babies had a longer time *in utero* to compensate for the effects of exposure, which might explain why smaller effects were observed in term births than in moderately preterm births.

Our estimate for the decrease in birth weight associated with entire pregnancy PM_{10} exposure (for an increase of 10 $\mu g/m^3$: 24.0 g, 95% CI 20.9 to 27.2) is considerably higher than estimates from previous studies. A meta-analysis including 7 studies showed a significant decrease in birth weight of 16.8 g (95% CI 13.3 to 20.2) as entire pregnancy PM_{10} exposure increased with 20 $\mu g/m^3$.[108] Dadvand *et al*.[106] combined effect estimates of 14 study centers established all over the world and observed a significant decrease in birth weight of 8.9 g (95% CI 4.6 to 13.2) for each 10 $\mu g/m^3$ increase in entire pregnancy exposure to PM_{10}. However, a pooled analysis of data from 14 population-based mother-child cohorts

in 12 European countries showed no significant association between PM_{10} exposure and birth weight (for an increase of 10 μg/m^3: -8 g, 95% CI -19 to 3).[121]

For SGA our results are consistent with a recent meta-analysis which found a positive association between SGA and entire pregnancy exposure to particulate matter with diameter less than 2.5 μg/m^3 ($PM_{2.5}$) (OR for an increase of 10 μg/m^3: 1.15, 95% CI 1.10 to 1.20).[122] For $PM_{10,}$ results of previous studies are inconsistent. One study reported a significant association between SGA and PM_{10} in the first gestational month (OR for an increase of 10 μg/m^3: 1.19, 95% CI 1.06 to 1.33) in Teplice.[28] Another study showed an association between SGA and exposure to PM_{10} in the second trimester, with an OR of 1.01 (95% CI 1.00 to 1.04) for a 1 μg/m^3 increase.[123] Hannam and colleagues[124] found a significant association between SGA and entire pregnancy PM_{10} exposure (OR for an increase from the 1st to the 4th quartile: 1.14, 95% CI 1.01 to 1.29). Other studies[125, 126] did not find significant associations between PM_{10} exposure and the risk for SGA.

The variability among study results may be due to differences in confounder adjustments, exposure assessment, study populations, and PM_{10} composition. Since the chemical composition of PM_{10} depends on the environmental air pollution sources, it may vary both spatially and temporally. The variety of sources causing the formation of PM_{10} may also emit other air pollutants such as sulphur dioxide (SO_2) and NO_2, which may have contributed to the observed effects. Also, as we found effect modification by variables such as parity, maternal age, parental education, and gestational duration, a different distribution of these or other variables between the studied populations could play a role in the observed differences in effect estimates.

We did not only consider exposure during pregnancy but also periconceptional exposure because air pollution may affect sperm cells and ova through genetic or epigenetic mechanisms,[127] and because placental methylation status at birth has been found to depend on exposures around implantation.[128] These early life exposure windows were significantly associated with both outcomes. The high correlation between entire pregnancy and trimester exposures hampers the identification of the time window in which the fetus is most vulnerable to air pollution. However, the observed effects tend to be higher for exposure in the first trimester than for exposure late in pregnancy (third trimester and last month),

especially for SGA and for moderately preterm births. The stronger association observed for entire pregnancy exposure compared with other time windows might indicate the importance of chronic exposure. However, because longer time windows typically have a smaller range and lower variability in exposure values than shorter windows, a 10 $\mu g/m^3$ increase in average PM_{10} for the entire pregnancy is relatively larger than the same increase for a shorter exposure window.

Some other studies investigated the shape of the association between adverse birth outcomes and ambient air pollution. Some conducted a linear analysis after an initial exploration of the shape.[120, 129] Ha *et al.*[110] found a significant association between birth weight and air pollution (SO_2, NO_2, total suspended particles and carbon monoxide) exposure in the first trimester. They allowed for nonlinear associations but concluded that the relations were relatively linear, without thresholds for concentrations of the pollutants. Similar to our study, Ballester *et al*.[109] showed a nonlinear association between birth weight and NO_2 exposure. However, we found a flattening out of the slope of the association between birth weight and PM_{10} at higher concentrations (above 30-40 $\mu g/m^3$), whereas they showed an increase in slope at higher NO_2 concentrations. A decrease in the effect of exposure at higher exposure levels was also observed for outcomes such as cardiovascular mortality,[130] lung and bladder cancer,[131] and respiratory epithelium integrity.[132] Weaker cardiovascular effects at higher particulate matter exposure may be caused by the saturation of underlying biochemical and cellular processes with small doses of harmful components.[133] Such saturation might also be the cause of the non-linear association in the current study.

The long study period and the large study population of nearly 530,000 newborns, including 30,982 preterm births, is a major strength of our study and enabled the investigation of effect modification by gestational age and many other factors, while maintaining sufficient statistical power. We observed effect modification by a number of factors including parity, maternal age, and parental education. The higher susceptibility observed for parents with lower education levels is consistent with previous observations.[134] Further, we observed a stronger effect for babies from young mothers (<25 years) compared to older mothers. Finally, we found higher effect estimates for higher parity newborns (≥2) than for firstborns. This is compatible with the hypothesis of Ritz and Yu[135] that exposure misclassification

is lower for parous mothers who tend to stay more at home to care for their other children.

Although we controlled for a number of potential confounders, we did not have information on some known important risk factors for birth weight and SGA. Birth certificates did not provide any indication of maternal nutrition, drinking and smoking behavior. On the other hand, a large number of important covariates was taken into account: year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence, apparent temperature, infant's sex, and gestational age. It is reasonable to assume that social economic indicators partly account for nutrition and lifestyle.[134]

Another potential source of bias in our analyses is exposure misclassification. We used interpolated ambient PM_{10} estimates at the level of the municipality (average size of 43.9 km²) as a proxy for individual exposure since home addresses were not available. However, this has several drawbacks. First, air pollution levels vary locally such that pregnant women living close to a major roadway or other pollution sources might be more heavily exposed than women living further away. Secondly, we only considered outdoor PM_{10} exposure, although indoor PM_{10} levels might add considerably to the overall burden of exposure for some mothers. A relevant indoor air pollution sources is environmental tobacco smoke.[136-138] Exposure misclassification might also be caused by pregnant women spending large amounts of time outside their municipality of residence.[139] Further, maternal residence is registered at the time of birth and women might have moved to another region during pregnancy. Therefore, misclassification is expected to be smallest later in pregnancy.[107] The date of conception in our study was estimated based on last menstruation and ultrasounds. An incorrect date of conception is more likely to affect exposure averaged over smaller time windows, such as the two weeks around and after conception. Finally, the exclusion of newborns with missing data could bias the results. Nevertheless, sensitivity analyses showed that our estimates were not altered by exclusion of subjects with missing data.

We expect that potential errors in PM_{10} exposure estimates are more or less the same for different levels of PM_{10} (non-differential misclassification), resulting in an underestimation of effect estimates. In a study of Ostro and colleagues[140]

adjustment of exposure estimates for time-activity pattern information, such as time spent outdoors, led to a 43% increase in the estimated effect of air pollution, suggesting non-differential misclassification if exposure estimates were based on fixed-site monitoring stations. Moreover, Wilhelm and Ritz[141] found stronger effects of air pollution (CO, PM_{10}, and $PM_{2.5}$) exposure if they limited their analysis to women living within 1 mile of a monitoring station. This indicates that our estimated effects of prenatal PM_{10} exposure are likely to be underestimated.

Conclusion

Our study findings indicate that, at PM_{10} levels below current air quality standards, prenatal exposure to particulate air pollution reduces birth weight and increases the risk of babies being small-for-gestational-age, not only among infants born at term, but even more strongly among babies born between 32 and 36 weeks of pregnancy. Assuming causality, 24% of moderately preterm SGA newborns and 13% of term SGA newborns could be prevented if average PM_{10} exposure during pregnancy was decreased to 20 $\mu g/m^3$.

Authors' contributions

E Winckelmans contributed to the design of the study in close collaboration with TS Nawrot and B Cox. E Martens collected and provided the data. Air pollution modelling was performed by F Fierens. E Winckelmans performed all statistical analyses and wrote the first draft of the manuscript in cooperation with B Cox. All authors were involved in the revision of the manuscript.

Funding

The study is part of the policy research center of Environment Health, commissioned and financed by the Ministry of the Flemish Community (Department of Economics, Science and Innovation; Flemish Agency for Care and Health; and Department of Environment, Nature and Energy), and co-financed by the EU Program "Ideas" (ERC-2012-StG 310898). The medical ethics committee of the Hasselt University approved the research protocol, and the research was

conducted in full accordance with the World Medical Association Declaration of Helsinki. EW and BC have a BOF PhD-fellowship (Bijzonder Onderzoeksfonds Hasselt University).

Supplementary material

Table S1. Association between birth weight and maternal exposure to PM_{10} in different time windows during pregnancy, comparison of main analysis and sensitivity analyses without adjustment for covariates with missing values

	Change in birth weight [g]		
		Sensitivity analyses	
	Main analysis†‡	Final study population‡	Total study population§
>36 weeks			
Entire pregnancy	-24.0 (-27.2 to -20.9)*	-27.8 (-30.9 to -24.7)*	-29.6 (-32.4 to -26.9)*
Two wks around conception	-4.0 (-5.5 to -2.8)*	-4.9 (-6.3 to -3.5)*	-5.7 (-7.0 to -4.5)*
Two wks after conception	-5.0 (-7.0 to -3.6)*	-5.7 (-7.1 to -4.3)*	-6.6 (-7.9 to -5.3)*
First trimester	-14.8 (-17.4 to -12.3)*	-17.7 (-20.2 to -15.1)*	-19.8 (-22.1 to -17.6)*
Second trimester	-16.6 (-19.1 to -14.0)*	-18.4 (-20.9 to -15.9)*	-19.2 (-21.4 to -16.9)*
Third trimester	-14.8 (-17.2 to -12.4)*	-16.5 (-18.9 to -14.0)*	-17.7 (-19.9 to -15.5)*
Last month	-8.8 (-10.6 to -7.0)*	-9.9 (-11.7 to -8.0)*	-10.9 (-12.5 to -9.2)*
32-36 weeks			
Entire pregnancy	-39.0 (-51.5 to -26.4)*	-40.3 (-52.8 to -27.8)*	-38.7 (-49.7 to -27.7)*
Two wks around conception	-13.9 (-19.5 to -8.2)*	-13.4 (-19.1 to -7.8)*	-11.4 (-16.4 to -6.3)*
Two wks after conception	-11.7 (-17.3 to -6.1)*	-11.7 (-17.3 to -6.1)*	-11.3 (-16.9 to -5.7)*
First trimester	-29.0 (-39.4 to -18.6)*	-28.9 (-39.3 to -18.5)*	-25.4 (-34.6 to -16.2)*
Second trimester	-26.0 (-36.5 to -15.5)*	-27.2 (-37.7 to -16.8)*	-30.5 (-39.7 to -21.2)*
Third trimester	-16.5 (-25.0 to -7.9)*	-16.8 (-25.3 to -8.2)*	-14.9 (-22.5 to -7.3)*
Last month	-10.2 (-17.7 to -2.7)*	-11.0 (-18.5 to -3.6)*	-10.8 (-17.5 to -4.1)*

Estimates (95% CI) are expressed as the change in birth weight for a 10 $\mu g/m^3$ increase in PM_{10} and are adjusted for year of birth, season of conception, parity, maternal age group, province of residence, apparent temperature, infant's sex and gestational age.

*P<0.05

†Additionally adjusted for marital status, maternal and paternal education, national origin of the mother

‡494,653 term births (>36 weeks) and 27,912 moderately preterm births (32-36 weeks)

§609,605 term births (>36 weeks) and 34,958 moderately preterm births (32-36 weeks)

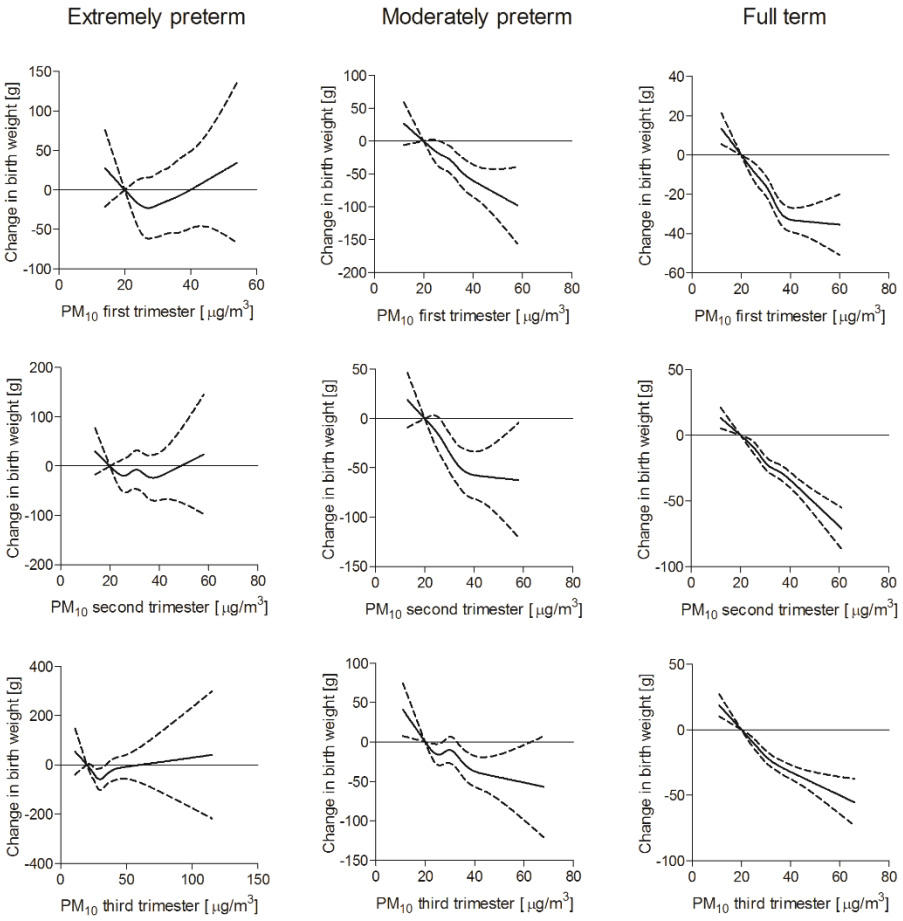

Figure S1. Shape of the association between birth weight and maternal PM_{10} exposure during each of the three trimesters of pregnancy, by gestational age group. Estimates (solid line) and 95% CI (dashed lines) represent the change in birth weight relative to the reference value of 20 μg/m^3. PM_{10} exposure was modeled using a natural cubic spline with 4 degrees of freedom and estimates are adjusted for year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence, apparent temperature, infant's sex, and gestational age.

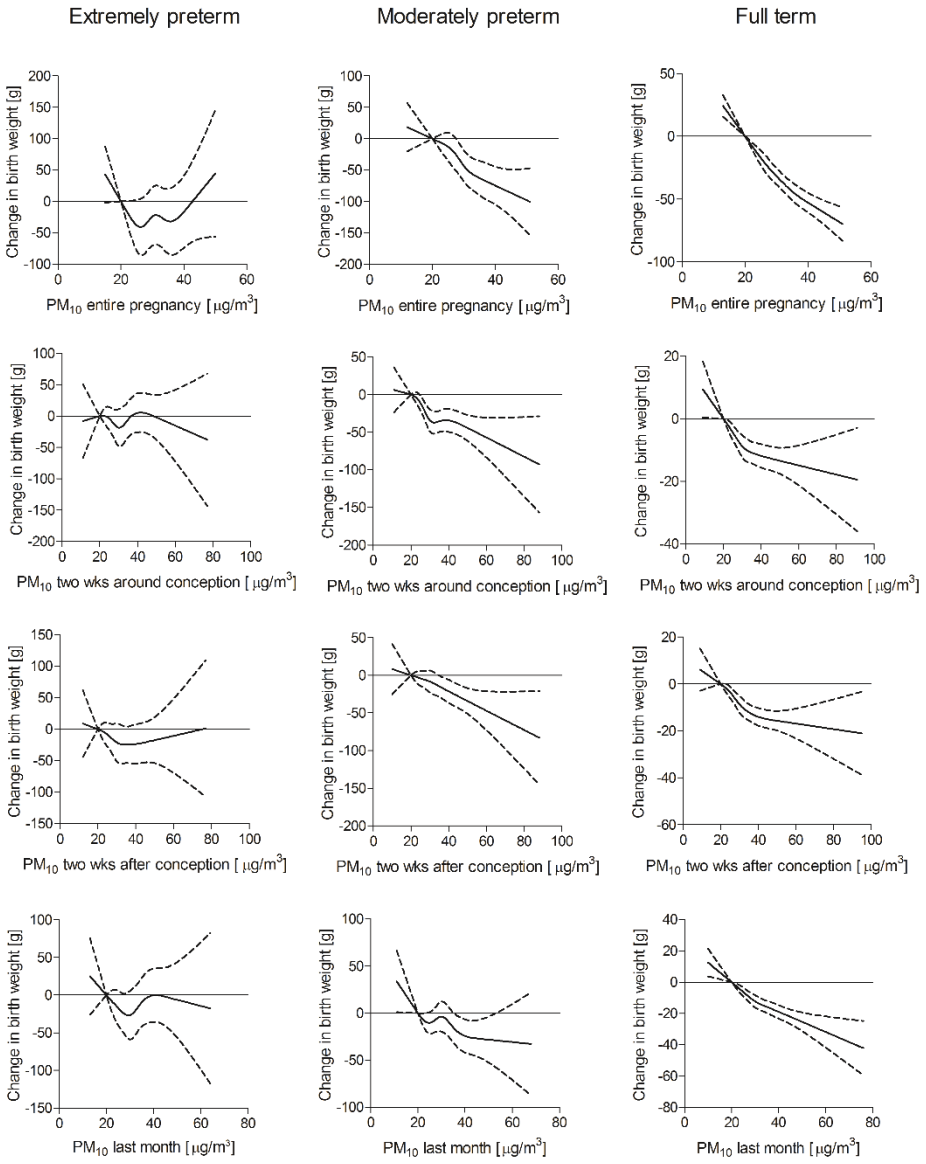

Figure S2. Shape of the association between birth weight and maternal PM_{10} exposure during entire pregnancy and during early and late pregnancy periods, by gestational age group. Estimates (solid line) and 95% CI (dashed lines) represent the change in birth weight relative to the reference value of 20 $\mu g/m^3$. PM_{10} exposure was modeled using a natural cubic spline with 4 degrees of freedom and estimates are adjusted for year of birth, season of conception, parity, maternal age group, marital status, maternal and paternal education, national origin of the mother, province of residence, apparent temperature, infant's sex, and gestational age.

Chapter 3

NEWBORN SEX-SPECIFIC TRANSCRIPTOME SIGNATURES AND GESTATIONAL EXPOSURE TO FINE PARTICLES: FINDINGS FROM THE ENVIR*ON*AGE BIRTH COHORT

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Environmental Health 2017; 16:52

Abstract

Background: Air pollution exposure during pregnancy has been associated with adverse birth outcomes and health problems later in life. We investigated sex-specific transcriptomic responses to gestational long- and short-term exposure to particulate matter with a diameter < 2.5 µm ($PM_{2.5}$) in order to elucidate potential underlying mechanisms of action.

Methods: Whole genome gene expression was investigated in cord blood of 142 mother-newborn pairs that were enrolled in the ENVIR*ON*AGE birth cohort. Daily $PM_{2.5}$ exposure levels were calculated for each mother's home address using a spatial-temporal interpolation model in combination with a dispersion model to estimate both long- (annual average before delivery) and short- (last month of pregnancy) term exposure. We explored the association between gene expression levels and $PM_{2.5}$ exposure, and identified modulated pathways by overrepresentation analysis and gene set enrichment analysis.

Results: Some processes were altered in both sexes for long- (e.g. DNA damage) or short-term exposure (e.g. olfactory signaling). For long-term exposure in boys neurodevelopment and RhoA pathways were modulated, while in girls defensin expression was down-regulated. For short-term exposure we identified pathways related to synaptic transmission and mitochondrial function (boys) and immune response (girls).

Conclusions: This is the first whole genome gene expression study in cord blood to identify sex-specific pathways altered by $PM_{2.5}$. The identified transcriptome pathways could provide new molecular insights as to the interaction pattern of early life $PM_{2.5}$ exposure with the biological development of the fetus.

Introduction

Changes in the transcriptome biology during fetal development can contribute to disease susceptibility. The fetal developmental period is known to be highly sensitive to environmental stressors causing alterations at different omic levels which may result in increased risk of disease in adulthood.[142-144] It has been hypothesized that specific transcriptome profiles in response to gestational exposure to fine particulate matter (PM) may not only act as signatures of exposure but could also be potentially prognostic for exposure-related health outcomes later in life.

Several observational studies corroborated the relationship between PM air pollution and adverse birth outcomes, such as decreased fetal growth[22, 105, 145] and preterm birth[146, 147]. Furthermore, perinatal physiological parameters like newborn systolic blood pressure were found to be associated with PM exposure during gestation.[148] Gestational air pollution exposure may affect the fetus in two different ways: 1) indirectly, through mediation by inflammatory effects on the mother's cardiorespiratory system and 2) directly, after translocation of (ultra)fine particles via the mother's bloodstream to the placenta. Wick *et al.* demonstrated in an *ex vivo* human placental perfusion model that polystyrene particles with a diameter up to 240 nm are able to cross the placental barrier.[149]

There is suggestive evidence that prenatal air pollution exposure may be linked to various adverse effects later in life such as cognitive and behavioral changes,[150, 151] cancer,[152, 153] and respiratory ailments[154, 155]. In addition, some studies reported sex differences in air pollution-related adverse health effects.[156, 157] Penaloza and colleagues[85] showed that sex-specific effects of prenatal exposure to environmental stressors are not only attributed to hormonal but also to chromosomal differences. Another study reported sex-specific associations between persistent organic pollutants and cord sex hormones.[86]

PM air pollution is an omnipresent environmental risk factor for public health in large areas of the world, however, the impact of gestational exposure to PM air pollution on fetal transcriptome profiles has not been assessed so far. In order to elucidate potential molecular mechanisms underlying prenatal $PM_{2.5}$-induced adverse health effects, we investigated sex-specific transcriptomic responses in

cord blood as part of the early life exposome in the framework of the ENVIR*ON*AGE birth cohort.

Methods

Study population

Mother-child pairs were enrolled in the on-going ENVIR*ON*AGE birth cohort (ENVIRonmental influence *ON* early AGEing) following procedures previously approved by the Ethical Committee of Hasselt University and the East-Limburg Hospital (09/080U;B37120107805),[92] and complies with the Helsinki declaration. All participating mothers provided written informed consent. Cord blood samples were collected along with perinatal parameters such as birth date, gestational age, newborn's sex, birth weight and length. The mothers completed study questionnaires in the post-delivery ward to provide detailed information on maternal age, pre-gestational body mass index (BMI), maternal education, smoking status, alcohol consumption, place of residence, parity, and ethnicity of the newborn. Former-smokers were defined as those who had quit smoking before pregnancy. Smokers were those who continued smoking during pregnancy. Based on the native country of the newborn's grandparents we classified his/her ethnicity as European-Caucasian when two or more grandparents were European, or as non-European when at least three grandparents were of non-European origin. We asked the mothers whether they consumed alcohol during pregnancy. Maternal education was coded as "low" (no diploma or primary school), "medium" (high school) or "high" (college or university degree).

The ENVIR*ON*AGE birth cohort had an overall participation rate of 61%. The current study is based on a representative subgroup of the ENVIR*ON*AGE birth cohort including 150 newborns recruited from South-East-Limburg Hospital in Genk (Belgium) born between Friday 1200 hours and Monday 0700 hours from March 20th 2010 until March 9th 2014. The general characteristics of the mother-child pairs did not differ from all births in Flanders as to maternal age, education, parity, sex, ethnicity, and birth weight (See Table S1). Quality control of microarray data resulted in exclusion of four newborns. Of the remaining 146 newborns, we excluded four newborns for whom no prenatal exposure (lived

outside the study area) were available. This resulted in a final sample of 142 mother-child pairs.

Ambient $PM_{2.5}$ exposure assessment

For each mother's residential address, $PM_{2.5}$ was calculated using a spatial temporal interpolation method (Kriging) taking into account land cover data obtained from satellite images (CORINE land cover data set) for interpolating the pollution data collected in the official fixed-site monitoring network in combination with a dispersion model (IFDM) using emissions from line sources and point sources.[97-99] This model chain provides daily $PM_{2.5}$ values on a high resolution irregular receptor grid (maximum grid cell size of 25x25m). Overall, model performance was evaluated by leave-one-out cross-validation including 34 monitoring points for $PM_{2.5}$. In our study area, the interpolation tool explained more than 80% of the temporal and spatial variability.[99] We defined two exposure windows of interest i.e. long-term (annual average before delivery) and short-term (last month of pregnancy) exposure. Annual averages before delivery were preferred to gestational exposure since annual averages are independent of season of blood sampling, an important predictor of gene expression.[158] Moreover, maternal $PM_{2.5}$ exposure during the three months before conception may induce maternal changes that may indirectly affect conception and the fetus and is thus included in annual averages. One month was taken as a period reflecting short-term exposure. Complete information was obtained for the residential address during and before pregnancy. For those who moved during pregnancy (n = 19; 13.4%), we calculated the exposure allowing for the changes in address during this period.

Meteorological data including mean daily air temperature and relative humidity were measured at the federal official station and provided by the Belgian Royal Meteorological Institute (Brussels, Belgium). Apparent temperature was averaged over one week before delivery and categorized based on the 25th, 50th and 75th percentiles.

RNA isolation

Total RNA was isolated from whole blood collected in Tempus tubes (ThermoFisher Scientific, Waltham, MA, USA) using the Tempus Spin RNA Isolation kit (Life

Technologies, Paisley, UK) according to the manufacturer's instructions. RNA yields were determined using the NanoDrop Spectrophotometer (Isogen Life Sciences, De Meern, the Netherlands) and the quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Samples with RNA Integrity Number below 6 were excluded from further analysis. Samples were stored at -80°C until further processing.

Microarray preparation, hybridization and preprocessing

An aliquot of 0.2 µg total RNA was reverse-transcribed into cDNA, labeled with cyanine-3 following the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies) and hybridized onto Agilent Whole Human Genome 8 x 60K microarrays. Microarray signals were detected using the Agilent DNA G2505C Microarray Scanner (Agilent Technologies). Scan images were converted into TXT files using the Agilent Feature Extraction Software (Version 10.7.3.1, Agilent Technologies, Amstelveen, The Netherlands), which were imported in R 2.15.3 (http://www.r-project.org). An in-house developed quality control pipeline in R software was used to preprocess raw data as follows: local background correction, omission of controls, flagging of bad spots and spots with too low intensity, $\log_2$ transformation and quantile normalization using arrayQC. The R-scripts of the quality control pipeline and more detailed information on the flagging can be found at https://github.com/BiGCAT-UM/arrayQC_Module. Further preprocessing included removal of genes with more than 30% flagged data, merging of replicates based on the median, imputation of missing values by means of K-nearest neighbor imputation (K=15) and correction for batch effects using an empirical Bayes method.[159] For genes represented by multiple probes, only the probe with the largest interquartile range was considered. The final dataset used for statistical analyses contained 16,844 genes.

Data analysis

To study alterations in gene expression in association with long-term (one year before delivery) and short-term (one month before delivery) exposure, multivariable-adjusted linear regression was performed while accounting for gestational age, season of conception, averaged apparent temperature over the last week of pregnancy (categories: <4.4, 4.4-7.9, 7.9-14.1, >14.1 C°), parity

(first, second, higher-order birth), maternal age, smoking status (never, past or current smoker), maternal education (lower secondary or less, higher secondary, higher education), ethnicity of the grandparents (European-Caucasian, yes or no), pre-pregnancy BMI, newborn's sex, long- or short-term $PM_{2.5}$ exposure, and the interaction term between newborn's sex and exposure. The interaction term was included in the models based on previous evidence suggesting differential responses between both sexes to environmental stressors during the perinatal period. Also at gene expression level, several animal studies[85, 160-163] and an epidemiological study of Hochstenbach and colleagues[143] observed sex-specific responses to prenatal environmental stress. For each sex, fold changes were calculated for an increase in long-term $PM_{2.5}$ exposure of 5 $\mu g/m^3$ and for an increase of 10 $\mu g/m^3$ in short-term $PM_{2.5}$ exposure. A *P*-value smaller than 0.05 was considered significant. A principal component analysis was performed based on the significant genes (*P*-value <0.05) for long- and short-term exposure for both sexes. Partial correlation coefficients (R) were calculated between principal component scores and long- and short-term $PM_{2.5}$ exposure.

In a sensitivity analysis, we additionally adjusted for white blood cell (WBC) counts and the percentage of neutrophils. However, due to blood clotting, data on these two variables were missing for 31 newborns. Normally, at birth the amount of WBCs ranges from 9-30 x $10^3/\mu L$. One newborn was excluded due to an outlying WBC count (>35 x $10^3/\mu L$). We assumed data is "at least missing at random". Single stochastic regression imputation was performed in SAS using the FCS statement in proc MI. For the WBC counts and percentage of neutrophils, we included in the imputation model the covariates of the main model and, respectively the top three significant genes related to WBC counts and neutrophil percentage resulting from a complete case analysis.

Pathway analysis by ConsensusPathDB

Genes significantly (p <0.05) associated with $PM_{2.5}$ exposure were uploaded into the Online Overrepresentation Analysis Tool ConsensusPathDB (http://consensuspathdb.org/)[164] of the Max Planck Institute for Molecular Genetics, to identify pathways associated with $PM_{2.5}$ exposure. A *P*-value representing the pathway of smaller than 0.05 was considered significant.

Gene Set Enrichment Analysis

The GSEA (Gene Set Enrichment Analysis) software tool (MSigDB, version 5.0)[165, 166] was used to find pathways significantly correlated with $PM_{2.5}$ exposure. Genes were ranked by the log_2-fold change. Subsequently, an enrichment score was calculated reflecting the degree a pathway is enriched by highly ranked genes. The statistical significance was estimated using a gene set permutation test with false discovery rate (FDR) correction for multiple hypothesis testing.

Pathways with a *Q*-value (FDR adjusted *P*-value) below 0.05 and *P*-value smaller than 0.005 were considered significant. Significant pathways were visualized using plug-in EnrichmentMap of cytoscape 3.2.0 software (http://cytoscape.org)[167]. An overlap coefficient of 0.5 was applied as similarity cutoff.

Results

Table 1 shows demographic characteristics and perinatal traits of the mother-child group (*n* = 142). Mean maternal age was 29.3 years (range: 18-42 yr) and mean (SD) pre-gestational BMI was 24.2 (4.6) kg/m^2. Most women never smoked (*n* = 80), 36 women stopped smoking before pregnancy, whereas 26 mothers reported to continue smoking during pregnancy (on average 8.6 cigarettes/day). More than 80% of the mothers never used alcoholic beverages during pregnancy. The newborns, among them 76 girls (53.5%), had a mean gestational age of 39.7 weeks (range, 35.9-41.1) and comprised 70 primiparous and 59 secundiparous newborns. About 90% of the newborns were Europeans of Caucasian ethnicity and their mean (SD) birth weight was 3454 (431) g. Maternal exposure to $PM_{2.5}$ over one year (long-term) and one month (short-term) preceding delivery averaged 16.0 (range: 11.8-20.6) and 13.3 (range: 6.5-34.8) µg/m^3 respectively.

Gestational $PM_{2.5}$ exposure and differential gene expression

A histogram of the percentage of genes associated with each of the covariates included in the model (*P*-value <0.05) is given in Figure S1. The effect of long-term gestational $PM_{2.5}$ exposure (annual average before delivery) on gene expression in cord blood revealed major differences between girls and boys. A total of 1269 (7.5%) genes showed a significant interaction between fine particle air pollution and the sex of the newborn. For girls and boys, this study identified

respectively 724 and 1358 genes which were significantly associated with long-term gestational $PM_{2.5}$ exposure. Among these genes, 75 were differentially expressed in both boys and girls (see Table S2). Supplemental Table S3 represents the top ten significant genes for boys and girls separately and their fold changes for a 5 $\mu g/m^3$ increment in $PM_{2.5}$ exposure.

Table 1. Demographic characteristics of the study population and exposure (*n*=142)

Characteristic	Mean (p10, p90) or *n* (%)
Mothers	
Age, yrs	29.3 (24.0, 34.0)
Pre-gestational BMI, kg/m^2	24.2 (19.5, 30.5)
Education	
Low	15 (10.6%)
Medium	50 (35.2%)
High	77 (54.2%)
Smoking status	
Never-smoker	80 (56.3%)
Former-smokers	36 (25.4%)
Smokers	26 (18.3%)
Alcohol consumption	
No	119 (83.8%)
Occasionally	23 (16.2%)
Parity	
1	70 (49.3%)
2	59 (41.5%)
≥3	13 (9.2%)
Newborns	
Sex	
Boys	66 (46.5%)
Season at conception	
Winter	38 (26.8%)
Spring	40 (28.2%)
Summer	37 (26.1%)
Autumn	27 (19.0%)
Ethnicity	
European-Caucasian	124 (87.3%)
Gestational age, wks	39.7 (38.3, 41.1)
Birth weight, g	3454 (2910, 4045)
Exposure	
Long-term[a] $PM_{2.5}$, $\mu g/m^3$	16.0 (13.9, 18.3)
Short-term[b] $PM_{2.5}$, $\mu g/m^3$	13.3 (8.0, 21.4)
Weekly apparent temp, °C	8.9 (2.4, 16.5)

[a] Annual average before delivery. [b] Last month of pregnancy. P: percentile

Figure S2A and S2B show the association of the first and second principal component score with long-term $PM_{2.5}$ exposure for girls and boys respectively. Both principal components were significantly associated with long-term $PM_{2.5}$ exposure in both girls (PC1: *P*-value <0.0001, R=0.51; PC2: *P*-value =0.03, R=-0.29) and boys (PC1: *P*-value =0.004, R=-0.40; PC2: *P*-value <0.0001, R=-0.63).

To identify potential short-term exposure effects on gene expression, we analyzed the microarray data while using the mean $PM_{2.5}$ exposure during the last month of pregnancy. We observed 432 (2.6%) genes of which the expression in boys and girls was differentially affected by exposure. For girls and boys, we identified 507 and 1144 genes respectively which were significantly associated with the last month of gestational $PM_{2.5}$ exposure. Of these, there were 55 significant genes in overlap between boys and girls (See Table S4). The top ten significant genes for each sex are given in Supplemental Table S5.

For boys, we found 180 genes significantly associated with both long- and short-term exposure, while 113 genes for girls.

Figure S2C and S2D show the association of the first and second (third) principal component score with short-term $PM_{2.5}$ exposure for girls and boys respectively. The first principal component was significantly associated with long-term $PM_{2.5}$ exposure in both girls (PC1: *P*-value =0.0005, R=0.43; PC2: *P*-value =0.20, R=0.17) and boys (PC1: *P*-value <0.0001, R=-0.58; PC2: *P*-value =0.28, R=0.16). For girls, the third principal component was significantly correlated with short-term $PM_{2.5}$ exposure (PC3: *P*-value =0.01, R=-0.31) and is therefore given on the y-axis in Figure S2C.

Overrepresentation analysis (ORA)

Newborn sex-specific $PM_{2.5}$ associated effects were further explored with overrepresentation analyses. The top 15 significant pathways with at least 15 measured genes and a total gene size of at most 500 genes are represented for both sexes in Table 2 and Table 3 for long- and short-term $PM_{2.5}$ exposure respectively. For each pathway, gene symbols and an indication of down- or up-regulation in association with $PM_{2.5}$ exposure are given for the significant genes. For pathways with the same contributing genes, only the most significant pathway is shown.

For girls, "Generic Transcription Pathway" and "Defensins" were the top most significant pathways in relation to long-term $PM_{2.5}$ exposure including 22% and 79% down-regulated genes respectively (Table 2). Both α- and β-defensins, involved in host defense and chronic inflammatory responses, were deregulated by long-term $PM_{2.5}$ exposure. Among the 11 measured genes specifically encoding defensin peptides, 9 were down-regulated. Other significant pathways were related to DNA damage response, cancer, signaling transduction, scavenging, and the extracellular matrix.

For boys, the "Tumor necrosis factor (TNF) receptor signaling pathway" was most significantly associated with long-term $PM_{2.5}$ exposure (Table 2). Other top significant pathways were mostly involved in the immune response or were related to cancer or the nervous system. Long-term $PM_{2.5}$ was associated with lower expression of various genes of the ephrin family [e.g. ephrins (*EPH*) and EPH-related receptors (*EFN*)] and members of the Roundabout (ROBO) family [e.g. *ROBO2* and *ROBO3*].

For the pathways "Oncogene Induced Senescence", "TP53 Network", and "Bladder Cancer", we observed a down-regulation of tumor protein p53 (*TP53*) and an increase of Mouse double minute 2 homolog (*MDM2*) expression, an important inhibitor of TP53 transcriptional activation.

For girls, overrepresentation analysis for short-term $PM_{2.5}$ exposure revealed pathways related to transcriptional regulation, immune response, embryonic development, cardiovascular system, and response to DNA damage (Table 3).

For boys, the top significant pathway for short-term $PM_{2.5}$ exposure was "Lidocaine (Local Anaesthetic) Action Pathway" which contains gene encoding voltage-gated sodium channels in peripheral neurons (Table 3). Other significant pathways were "Hedgehog ligand biogenesis" important for embryonic development, "Tricarboxylic acid (TCA) cycle" responsible for energy production, and "Neuroactive ligand-receptor interaction - Homo sapiens (human)" including several neurotransmitter receptor encoding genes which are negatively associated with short-term $PM_{2.5}$ exposure.

Table 2. Top 15 overrepresented pathways associated with long-term $PM_{2.5}$ exposure for girls and boys

Sex	Pathway	Effective/ total size	# ↓ genes	Contributing genes	*P*-value
Girls					
	Generic Transcription Pathway[a]	367/478	80	Top 5 out of 33 significant genes: ZNF124↑; MED16↑; KRBA1↑; ZNF205↓; ZNF720↑	3.0E-06
	Defensins	19/53	15	ART1↓;DEFA3↓; DEFB1↓;DEFB128↑;DEFA4↓	5.7E-04
	Binding and Uptake of Ligands by Scavenger Receptors[a]	28/43	15	APOA1↓; HPR↓; HP↓; HBA2↑; FTL↑	3.6E-03
	agrin in postsynaptic differentiation	39/47	18	EGFR↑; PTK2↑; UTRN↑; ITGB1↑; CHRM1↓	1.5E-02
	JAK-STAT[a]	39/43	15	PTK2↑; ESR1↓; ZAP70↑;PDK1↑; ITGB1↑	1.5E-02
	ATM Signaling Pathway[a]	15/18	7	ATM↑;ATF2↑;RAD51↓	1.7E-02
	Integrated Pancreatic Cancer Pathway	141/165	62	SERPINB10↓;CAMP↓;RAD51↓;TYMS↓;INHBA↓; NTRK1↓;ATM↑;EGFR↑	1.8E-02
	Transcriptional misregulation in cancer - Homo sapiens (human)	146/179	73	CEBPE↓; CDKN2C↓; EWSR1↑; DEFA3↓; HIST1H3J↓; PTK2↑; ASPSCR1↓; MPO↓; NTRK1↓; ELANE↓; ATM↑	2.3E-02
	BARD1 signaling events	29/29	17	RAD51↓;ATM↑;EWSR1↑;UBE2D3↑	2.3E-02
	Gastric cancer network 2	29/32	9	CACYBP↑;AHCTF1↑;EGFR↑;BRIX1↑	2.3E-02
	Extracellular matrix organization	167/275	92	ITGB1↑; ELANE↓; MMP17↓; LTBP3↑; PLOD1↑; CTSG↓; CEACAM1↓; MMP8↓; CEACAM6↓; CEACAM8↓; COL17A1↓	2.5E-02
	Urokinase-type plasminogen activator (uPA) and uPAR-mediated signaling	32/45	15	CTSG↓;EGFR↑;ELANE↓;ITGB1↑	3.2E-02
	Downregulation of SMAD2/3:SMAD4 transcriptional activity	20/21	3	UBA52↑; TGIF2↑; PPM1A↑	3.8E-02
	JAK-STAT-Core	67/104	29	IL11RA↑; IL12RB1↑; STAT4↑; MPL↑; EGFR↑; IL6ST↑	4.1E-02
Boys					
	TNF receptor signaling pathway	44/48	29	IKBKB↑; MAP4K5↑; TAB2↑; TAB1↓; MAP2K3↑; TNIK↓; TNF↓; IKBKG↑; GNB2L1↓	4.8E-03
	Mercaptopurine Action Pathway	38/47	21	ATIC↓; PAICS↓; TPMT↓; APRT↓; ITPA↓; ADA↓; ABCC4↓; ADSL↓	6.5E-03
	Primary immunodeficiency - Homo sapiens (human)	32/36	25	ICOS↓; ORAI1↓; CD40LG↓; IKBKG↑; ADA↓; CD3D↓; LCK↓	8.6E-03
	the co-stimulatory signal during t-cell activation	18/21	13	CTLA4↓; ICOS↓; CD3D↓; LCK↓; ITK↓	9.0E-03
	FCERI mediated NF-kB activation[a]	19/63	13	IKBKB↑; TAB2↑; TAB1↓; RASGRP1↓; IKBKG↑	1.1E-02
	p73 transcription factor network	68/81	38	GNB2L1↓; PFDN5↑; PLPP1↓; UBE4B↑; TP73↓; BAK1↓; FOXO3↑; ADA↓; MDM2↑; BIN1↓; MYC↓	1.2E-02
	Axon guidance - Homo sapiens (human)*	88/127	57	EPHB2↓; EPHB3↓; ROBO2↓; PPP3R1↑; ROBO3↓; EFNA4↓; EFNA3↓; ROCK1↑; EPHA1↓; ITGB1↑; RGS3↓; ABLIM1↓; SEMA4C↓	1.4E-02
	Thiopurine Pathway, Pharmacokinetics/Pharmacodynamics	28/32	22	PRPS1↓; TPMT↓; NT5E↓; ITPA↓; ADA↓; ABCC4↑	1.6E-02
	T cell receptor signaling pathway - Homo sapiens (human)	91/104	59	DLG1↑; CTLA4↓; ICOS↓; RASGRP1↓; CD40LG↓; ITK↓; PPP3R1↑; IKBKB↑; TNF↓; CDK4↓; IKBKG↑; CD3D↓; LCK↓	1.8E-02

Table 2. Top 15 overrepresented pathways associated with long-term $PM_{2.5}$ exposure for girls and boys (continued)

Sex	Pathway	Effective/ total size	# ↓ genes	Contributing genes	*P*-value
	Oncogene Induced Senescence[a]	29/31	13	TP53↓; E2F2↑; CDK4↓; TFDP1↑; MDM2↑; AGO3↑	1.9E-02
	Regulation of nuclear beta catenin signaling and target gene transcription	64/81	39	TCF7↓; HDAC2↓; TBL1XR1↑; AES↓; CAMK4↓; TNIK↓; APC↑; MYC↓; LEF1↓; AXIN2↓	2.1E-02
	TP53 Network	15/18	7	MDM2↑; TP53↓; MYC↓; TP73↓	2.2E-02
	Bladder Cancer	23/26	14	CDK4↓; TYMP↓; MDM2↑; TP53↓; MYC↓	2.6E-02
	Stimuli-sensing channels	68/102	38	TRPV6↓; CLCN3↑; WNK2↓; TRPM5↓; CLCN7↑; ANO10↓; TPCN1↑; BEST4↓; WWP1↓; WNK1↑	3.0E-02
	Amyotrophic lateral sclerosis (ALS) - Homo sapiens (human)	41/51	23	NEFH↓; PPP3R1↑; TOMM40↓; TNF↓; BCL2L1↑; MAP2K3↑; TP53↓	3.2E-02

↓ genes: number of down-regulated genes. [a] Pathways that remain significant in the sensitivity analysis.

Table 3. Top 15 overrepresented pathways associated with short-term $PM_{2.5}$ exposure for girls and boys

Sex	Pathway	Effective /total size	# ↓ genes	Contributing genes	*P*-value
Girls					
	mRNA Processing[a]	124/126	32	PTBP2↑; SRSF1↑; SFPQ↑; SNRNP40↑; CELF1↑; HNRNPU↑; TRA2B↑; SRSF6↑; HNRNPH1↑; PRPF40A↑	1.9E-03
	Ephrin signaling	16/22	5	NCK2↑; SDCBP↑; ARHGEF7↑	8.4E-03
	Ectoderm Commitment Pathway[a]	87/129	30	PDE7A↑; SDCBP↑; MZF1↑; C1GALT1↑ NF2↑; OGT↑; TSC22D1↑	9.1E-03
	IL-4 Signaling Pathway[a]	52/53	27	IKBKB↑; PTPN11↑; IL2RG↓; ATF2↑; RPS6KB1↑	1.3E-02
	Physiological and Pathological Hypertrophy of the Heart[a]	20/24	8	IL6ST↑; CAMK2D↑; PPP3CB↑	1.6E-02
	miR-targeted genes in lymphocytes - TarBase	362/482	108	Top 5 out of 17 genes: MBNL1↑; SUCLG2↑; TGFBR2↑; GTPBP3↑; DMTF1↑	1.9E-02
	Basal transcription factors - Homo sapiens (human) [a]	39/45	13	TAF8↑; GTF2H2C_2↑; TAF1↑; TAF11↑	2.1E-02
	Spliceosome - Homo sapiens (human) [a]	127/130	33	HNRNPU↑; PRPF40A↑; RBM25↑; SNRNP40↑; THOC1↑; SRSF1↑; SRSF6↑; TRA2B↑	2.2E-02
	Activated TLR4 signaling[a]	110/120	47	ATF2↑; SIGIRR↑; IL6ST↑; PTPN11↑; IKBKB↑; IRF3↑; UBE2D3↑	2.9E-02
	Insulin Pathway	44/47	13	RPS6KB1↑; PTPN11↑; NCK2↑; EXOC7↑	3.0E-02
	Salmonella infection - Homo sapiens (human)	68/86	38	PFN1↓; RAB7A↓; DYNC1H1↑; WAS↓; PKN2↑	3.7E-02
	Amphetamine addiction - Homo sapiens (human) [a]	48/68	21	PPP3CB↑; CAMK2D↑; ATF4↑; ATF2↑	4.0E-02
	Generic Transcription Pathway	367/478	91	Top 5 out of 16 genes: ZNF625↑; ZNF37A↑; ZNF419↑; ZNF205↓; ZNF12↑	4.0E-02
	Direct p53 effectors[a]	123/147	47	PMS2↑; KAT2A↑; BNIP3L↑; TGFA↓; PIDD1↑; AIFM2↑; HIC1↓	4.9E-02
Boys					
	Lidocaine (Local Anaesthetic) Action Pathway[a]	19/31	13	CYP3A4↓; CACNA2D2↓; ATP1A4↑; ATP1B1↑; ATP1B3↑; ADRA1A↓	9.9E-04
	Signaling events mediated by PRL[a]	20/23	4	CDK2↑; BCAR1↓; RABGGTA↑; PTP4A3↑; ROCK1↑; ITGB1↑	1.3E-03
	Protein processing in endoplasmic reticulum - Homo sapiens (human)[a]	156/168	36	Top 5 out of 19 significant genes: ATF4↑; SEC31A↑; UBQLN3↓; UGGT1↑; CRYAB↓	6.2E-03
	Basigin interactions[a]	19/30	6	ATP1B3↑; SLC3A2↑; ATP1B1↑; CAV1↓; ITGB1↑	6.3E-03
	Morphine Action Pathway[a]	27/44	16	DNAJB11↑; CACNA2D2↓; ATP1A4↑; ATP1B1↑; ATP1B3↑; ADRA1A↓	6.9E-03
	mRNA Splicing - Major Pathway[a]	116/131	14	Top 5 out of 15 significant genes: SMC1A↑; PCBP1↑; PRPF8↑; SNRPA↓; CD2BP2↑	8.3E-03
	Validated transcriptional targets of AP1 family members Fra1 and Fra2	30/37	11	ATF4↑; TXLNG↑; LAMA3↓; NFATC3↑; USF2↑; CDKN2A↓	1.2E-02
	Maturity onset diabetes of the young - Homo sapiens (human)	15/25	14	NR5A2↓; PAX4↓; FOXA2↓; GCK↓	1.4E-02
	Hedgehog ligand biogenesis[a]	15/21	5	OS9↑; DISP2↓; P4HB↑; VCP↑	1.4E-02

Table 3. Top 15 overrepresented pathways associated with short-term $PM_{2.5}$ exposure for girls and boys (continued)

Sex	Pathway	Effective /total size	# ↓ genes	Contributing genes	*P*-value
	Salivary secretion - Homo sapiens (human) [a]	60/90	28	ADCY3↓; ADRA1A↓; NOS1↓; GUCY1A3↑; PRH2↓; ATP1A4↑; ATP1B1↑; ATP1B3↑; ATP2B3↓	1.5E-02
	Processing of Capped Intron-Containing Pre-mRNA[a]	147/162	18	Top 5 out of 17 significant genes: SMC1A↑; RANBP2↑; PCBP1↑; PRPF8↑; SNRPA↓	1.5E-02
	G. alpha (s) signaling events[a]	81/129	44	ADCYAP1R1↓; CALCA↓; PTH2↓; ADCY3↓; GNAZ↓; TSHB↓; INSL3↓; TAAR2↓; GHRHR↓; GLP2R↓; GNG13↓	1.6E-02
	Neuroactive ligand-receptor interaction - Homo sapiens (human)	164/275	102	GABRG2↓; GABRP↓; NTSR2↓; TAAR2↓; TSHB↓; CHRM5↓; ADCYAP1R1↓; GH1↓; GHRHR↓; HTR1B↓; ADRA1A↓; GLP2R↓; THRA↓; ADORA1↓; CHRNA2↓; LPAR1↑; OPRL1↓; GRM5↓	2.1E-02
	FOXM1 transcription factor network	34/42	10	CDK2↑; XRCC1↑; CENPF↑; NFATC3↑; TGFA↓; CDKN2A↓	2.1E-02
	TCA Cycle	17/17	4	FH↑; MDH2↑; OGDH↑; IDH2↑	2.2E-02

↓ genes: number of down-regulated genes. [a] Pathways that remain significant in the sensitivity analysis.

Gene Set Enrichment Analysis (GSEA)

Clusters of functional related pathways, modulated by long- and short-term $PM_{2.5}$ exposure, are presented in Figure S3 and S4 respectively. Each cluster is encircled and assigned a label. Table 4 and 5 list the cluster labels and the corresponding individual pathways which were significantly up- or downregulated by long- and short-term $PM_{2.5}$ exposure respectively. Table 4 shows the GSEA results for long-term exposure in girls which were consistent with the overrepresentation analysis for 1) the pathways "Defensins" and "Extracellular matrix organization", which both were down-regulated, and for 2) the pathways related to Transcription-SMAD2, 3, 4-TGFβ which were up-regulated. Additional pathways were related to the cell cycle ("FOXM1" and "Aurora B pathway") and pathways containing genes encoding histone peptides, ribosomal peptides, and olfactory receptors.

For boys, the top significant pathways modulated by long-term $PM_{2.5}$ exposure were all up-regulated (Table 4) and were related with cell cycle, plasminogen activation system (UPA-UPAR pathway), execution phase of apoptosis, Ras homolog gene family member A (RhoA) pathway, and regulation of gene expression by histone deacetylase (HDAC) class III. The 18 "leading edge genes" of the RhoA pathway included among others Diaphanous-Related Formin 1 (*DIAPH1*), Rho-Associated Coiled-Coil Containing Protein Kinase 1 (*ROCK1*), and *ROCK2* of which the gene products are effectors of RhoA. Two of these effectors, *ROCK1* and *DIAPH1* were significantly associated with long-term $PM_{2.5}$ exposure. Plasminogen activation system was also $PM_{2.5}$ sensitive in girls (Table 2).

For girls, GSEA results for short-term $PM_{2.5}$ exposure revealed significantly up-regulated pathways related to ribosomes and significantly down-regulated pathways related to the Rho pathway and olfactory signaling (Table 5). As found before in girls for long-term exposure, both olfactory signaling and ribosome related pathways were also significantly associated with short-term $PM_{2.5}$ exposure. The Rho pathway contained 12 "leading edge genes" including *RHOA*, *DIAPH1*, LIM domain kinase 1 (*LIMK1*), Cofilin 1 (*CFL1*), several members of the Rho guanine nucleotide exchange factors (ARHGEF) family, and genes encoding subunits of the Actin Related Protein 2/3 Complex. However, none of these genes were significantly associated with short-term $PM_{2.5}$ exposure.

For boys, there were 132 significantly up-regulated and 11 down-regulated pathways by short-term $PM_{2.5}$ exposure. Because of the large amount of significant pathways, Table 5 represents only the pathways with both *P*-value and *Q*-value smaller than 0.005. Most of the significant pathways were up-regulated and linked to the cell cycle or ribosomes. Other up-regulated pathways were related to the TCA cycle and DNA damage response including "BRCA1 Associated RING Domain 1 (BARD1) pathway" and "Ataxia Telangiectasia Mutated (ATM) pathway". The 23 "leading edge genes" of the BARD1 pathway included among others *BARD1*, Breast Cancer 1 Early Onset (*BRCA1*), and *ATM*. Note that "BARD1 pathway" and "ATM pathway" were also significantly associated with long-term $PM_{2.5}$ exposure in girls (Table 2). The RhoA pathway results were similar as those for long-term $PM_{2.5}$ exposure. *DIAPH1* and *ROCK1* were both significantly associated with short-term $PM_{2.5}$ exposure and contributed to the "leading edge genes". Down-regulated pathways were related to olfactory receptor signaling pathways.

Sensitivity analysis

It has been reported that air pollution exposure can induce changes in WBC counts in adults,[168, 169] and changes in cord blood cell distribution might influence the overall blood transcriptome profile. However, in our newborn cohort, we did not find a significant association between $PM_{2.5}$ exposure and WBC count and neutrophil percentage in cord blood. Nevertheless, in a sensitivity analysis we added WBC count and neutrophil percentage to the main model. For girls, 525 (72.5%) of the significant genes in the main analysis remained significantly associated with long-term $PM_{2.5}$ exposure after adjustment for WBC count and neutrophil percentage. Overrepresented pathways of the main analysis that remained significant in the sensitivity analysis are marked ([a]) (Table 2). For GSEA, pathways related to defensins, histones ("Amyloids"), extracellular matrix organization, and olfactory receptors remained in the top most significant pathways.

Table 4. Pathways modulated by long-term $PM_{2.5}$ exposure for girls and boys resulting from GSEA

Sex	Cluster label	Source: pathway	# genes	Direction of regulation
Girls	Aurora B pathway	PID: Aurora B pathway	36	DOWN
	Core matrisome	Matrisome: Naba core matrisome	142	DOWN
	Defensins	Reactome: defensins	18	DOWN
	Extracellular matrix organization			
		Reactome: extracellular matrix organization	49	DOWN
		Reactome: degradation of the extracellular matrix	18	
	FOXM1 pathway	PID: FOXM1 pathway	32	DOWN
	Histone related pathways			
		Reactome: amyloids	69	DOWN
		Reactome: RNA polymerase I promotor opening	54	DOWN
		KEGG: systemic lupus erythematosus	116	DOWN
	Olfactory signaling			
		KEGG: olfactory transduction	124	DOWN
		Reactome: olfactory signaling pathway	95	DOWN
	Porphyrin metabolism	KEGG: porphyrin and chlorophyll metabolism	25	DOWN
	Ribosome related pathways			
		Reactome: peptide chain elongation	83	UP
		KEGG: ribosome	85	
		Reactome: nonsense mediated decay enhanced by the exon junction complex	104	
	Transcription-SMAD2,3,4-TGFβ pathways			
		Reactome: generic transcription pathway	267	UP
		Reactome: downregulation of SMAD2, 3, SMAD4 transcriptional activity	18	
		Reactome: signaling by TGF-beta receptor complex	54	
Boys	Apoptotic execution	Reactome: apoptotic execution phase	43	UP
	Cell cycle			
		Reactome: cell cycle mitotic	290	UP
		Reactome: mitotic prometaphase	79	
		Reactome: DNA replication	176	

Table 4. Pathways modulated by long-term $PM_{2.5}$ exposure for girls and boys resulting from GSEA (continued)

Sex	Cluster label	Source: pathway	# genes	Direction of regulation
	HDAC class III	PID: HDAC class III pathway	22	UP
	UPA-UPAR pathway	PID: uPA uPAR pathway	30	UP
	RhoA pathway	PID: RhoA pathway	37	UP

For cluster containing more than 3 pathways, only the top 3 significant pathways are given.
gene: number of genes within a pathway. uPAR: Urokinase-type plasminogen activator (uPA) receptor; HDAC: histone deacetylase; FOXM1: forkhead box M1; RhoA: Ras homolog gene family member A; PID: Pathway Interaction Database; KEGG: Kyoto Encyclopedia of Genes and Genomes

Table 5. Pathways modulated by short-term $PM_{2.5}$ exposure for girls and boys resulting from GSEA

Sex	Cluster label	Source: pathway	# genes	Direction of regulation
Girls	Olfactory signaling	Reactome: olfactory signaling pathway	95	DOWN
	Rho pathway	BioCarta: Rho pathway	28	DOWN
	Ribosome related pathways			
		Reactome: nonsense mediated decay enhanced by the exon junction complex	104	UP
		KEGG: ribosome	85	
		Reactome: 3' UTR mediated translational regulation	102	
Boys	ATM pathway	PID: ATM pathway	18	UP
	BARD1 pathway	PID: BARD1 pathway	29	UP
	Cell Cycle			
		Reactome: DNA replication	176	UP
		Reactome: G2/M checkpoints	37	
		Reactome: cell cycle mitotic	290	
	ETC-TCA cycle			
		Reactome: TCA cycle and respiratory electron transport	113	UP
		Reactome: respiratory electron transport atp synthesis by chemiosmotic coupling and heat production by uncoupling proteins	79	
	M-calpain pathway	BioCarta: M-calpain pathway	21	UP
	Metabolism of mRNA and RNA			
		Reactome: metabolism of RNA	249	UP
		Reactome: metabolism of mRNA	204	
	Myc pathway	PID: Myc activ pathway	76	UP
	Olfactory signaling			
		KEGG: olfactory transduction	124	DOWN
		Reactome: olfactory signaling pathway	95	
	mRNA processing			
		Reactome: processing of capped intron containing pre mRNA	133	UP
		Reactome: mRNA processing	147	
	Response to elevated platelet cytosolic CA^{2+}	Reactome: response to elevated platelet cytosolic CA^{2+}	66	UP

Table 5. Pathways modulated by short-term $PM_{2.5}$ exposure for girls and boys resulting from GSEA (continued)

Sex	Cluster label	Source: pathway	# genes	Direction of regulation
	Ribosome related pathways			
		Reactome: translation	141	UP
		Reactome: SRP dependent cotranslational protein targeting to membrane	105	
		Reactome: 3' UTR mediated translational regulation	102	
	RhoA pathway	PID: RhoA pathway	37	UP
	Splicesome	KEGG: spliceosome	123	UP

For cluster containing more than 3 pathways, only the top 3 significant pathways are given.
genes: number of genes within a pathway. Rho: Ras Homolog gene family; TCA: tricarboxylic acid; ETC: electron transport chain; ATM: Ataxia Telangiectasia Mutated; BARD1: BRCA1 associated RING domain 1; Myc: v-myc avian myelocytomatosis viral oncogene homolog; PID: Pathway Interaction Database; KEGG: Kyoto Encyclopedia of Genes and Genomes

For boys, 773 (56.9%) of the significant genes in the main analysis remained significant after adjustment for WBC count and neutrophil percentage. GSEA confirmed our main findings with pathways related to the cell cycle (*Q*-value < 0.25 and *P*-value < 0.005) including "Mitotic M-M/G1 phases", "Cell cycle mitotic", and "Loss of Ninein-Like Protein (NLP) from mitotic centrosomes".

Short-term $PM_{2.5}$ exposure

For girls, 433 (85.4%) genes which significantly correlated with short-term $PM_{2.5}$ exposure in the main analysis were in overlap with the sensitivity analysis. Of the top 15 significant enriched pathways for short-term $PM_{2.5}$ exposure in girls (Table 3), nine pathways remained significantly overrepresented in the sensitivity analysis. No significant up-regulated pathways resulted from GSEA, however, ribosome related pathways had the most significant positive association with short-term $PM_{2.5}$ exposure. Pathways related to olfactory signaling remained significantly down-regulated.

For boys, 1055 (92.2%) of the significant genes in the main analysis remained significantly correlated with short-term $PM_{2.5}$ exposure in the sensitivity analysis. The most significant overrepresented pathway after adjustment for blood count was proteasome complex of which all ten contributing genes were up-regulated. Eight of these genes encoded proteasome subunits. Of the top 15 significant pathways in the main overrepresentation analysis, ten pathways remained significantly enriched in the sensitivity analysis (Table 3). GSEA revealed 134 significantly up-regulated and 13 down-regulated pathways. All pathways shown in Table 5 remained significant except the "M-calpain pathway".

Discussion

This is the first paper reporting neonate transcriptome signatures for long-term and short-term gestational exposure to PM. Although epidemiological studies are scarce, transcriptome alterations in early life may act in response to environmental exposures heralding adverse health outcomes later in life. At the gene level we observed in cord blood substantial differences in transcriptomic responses between newborn girls and boys in association with air pollution exposure during pregnancy. However, pathway analyses revealed alterations in the immune and DNA damage responses in both sexes for long-term exposure.

Considering short-term exposure (last month of pregnancy), significant pathways were identified for both girls and boys which were related to olfactory receptors, ribosomes, and DNA damage. For long-term exposure, we also found sex-specific pathways including "axon guidance" and "RhoA pathway" for boys, while olfactory receptor, cell cycle, ribosomal, and defensin-related processes were girl-specific. Sex-specific pathways associated with short-term exposure in boys included processes involved in synaptic transmission ("neuroactive ligand-receptor interaction") and mitochondrial energy production, and for girls immune response pathways. Table 6 gives an overview of these biological processes altered by gestational PM exposure.

Table 6. Overview of selected biological processes altered by gestational PM exposure

	Long-term				**Short-term**			
	Girls		**Boys**		**Girls**		**Boys**	
	ORA	GSEA	ORA	GSEA	ORA	GSEA	ORA	GSEA
Nervous system		SA	SA			SA		SA
Neurotransmission								
Neurodevelopment			SA					
Olfactory		SA				SA		SA
Cell		SA		SA		SA		SA
Cell cycle				(SA)↑				SA
Ribosomal proteins						(SA)↑		SA
Histone proteins		SA						
RhoA								SA
Mitochondrial energy producing processes								SA
Apoptosis								
Protective response	SA	SA	SA		SA			SA
DNA damage response	SA		SA		SA			SA
Defensins		SA						
Other immune	SA		SA		SA			

ORA, Overrepresentation Analysis; GSEA, Gene Set Enrichment Analysis
Gray: $PM_{2.5}$-related processes in the main analysis. SA: processes that remained significant in the sensitivity analysis. (SA)↑: most significant up-regulated pathways in the sensitivity analysis.

Neural pathways

We suggest that the observed inverse association between gene expression of olfactory receptors could be an early marker of the effects of fine particle air pollution on the central nervous system. An association between air pollution exposure and olfactory dysfunction has been suggested to be involved in the development of various diseases such as Alzheimer and Parkinson's disease.[170] Importantly, the functional role of gene expression of olfactory receptors in blood parallels severity of head injury as indicated in patients suffering of traumatic brain injury.[171]

Besides olfactory receptor signaling, we identified other neurological pathways affected by long- and short-term $PM_{2.5}$ exposure in boys. Long-term exposure down-regulated the expression of ROBO, EPH and EFN members which are essential for axon guidance during neurodevelopment. Short-term $PM_{2.5}$ exposure altered expression of "Neuroactive ligand-receptor interaction - Homo sapiens (human)" gene members including several types of neurotransmitter receptor encoding genes such as gamma-aminobutyric acid (GABA) receptors, cholinergic and glutamate receptors. Interestingly, all these contributing genes were negatively correlated with $PM_{2.5}$ exposure. In mice, decreased expression of ionotropic glutamate receptor subunit in the hippocampus of offspring was shown following gestational exposure to benzo(a)pyrene.[172] In rats, exposure to cigarette smoke showed a dose-dependent decrease of GABA B receptor, 1 mRNA expression in the hippocampus.[173] Changes in neurotransmitter receptor expressions early in life are predictive for cognitive dysfunction and behavior deficits later in life.[174]

Genotoxic pathways

In adults, the increased risk in lung cancer associated with ambient air pollution is suspected to be linked to genotoxic chemicals absorbed on PM, more specifically polycyclic aromatic hydrocarbons (PAH),[175] and toxic metals e.g. cadmium[176]. Fetuses are more susceptible to carcinogenic exposures due to their rapid cell proliferation and differentiation, greater absorption and retention, immature immune system, and decreased capacity of detoxification, DNA repair or apoptotic.[19, 20] Micronuclei, a validated biomarker of cancer risk, are extranuclear bodies originating from dividing cells that are formed by chromosomal breakage

and/or whole chromosome loss.[177] A Danish birth cohort showed that micronuclei frequencies, measured in cord blood, were elevated among newborns whose mothers lived in high-traffic-density areas.[178] In our study, we identified several pathways that may underlie the carcinogenic potential of air pollution in early life. "ATM" and "BARD1" pathways were significantly modulated by $PM_{2.5}$ exposure for short-term exposure in boys and long-term exposure in girls. These pathways play a central role in the response to DNA damage and may be important in the potential of $PM_{2.5}$ to induce genotoxic stress. Jiang *et al.* found elevated *ATM* expression in esophageal squamous cell carcinoma specimens of smokers compared to non-smokers.[179]

Other pathways related to DNA damage which were significantly associated with long-term $PM_{2.5}$ exposure were "p73 transcription factor network", "Oncogen induced Senescence", and "*TP53* network" in boys only. At the gene level the up-regulation of *MDM2*, a negative regulator of *TP53*, is in line with the inverse association of long-term $PM_{2.5}$ exposure and *TP53* expression and its family member *TP73*. In contrast to our observations, Rossner *et al.* reported positive associations between p53 protein plasma levels and personal PAH exposure in city policemen and bus drivers at work.[180]

Expression of these DNA damage responsive genes seem to be affected by $PM_{2.5}$ exposure in a time dependent manner. It is plausible that deregulated gene expression of key players of the response to DNA damage, as a consequence of fine particle air pollution exposure, may increase the susceptibility to develop cancer and other diseases later in life.

Hypertension related pathways

The positive association between expression of gene members of the RhoA pathway, which are important for cytoskeleton organization, and gestational long- and short-term $PM_{2.5}$ exposure for boys supports the idea that air pollution can activate the Rho/ROCK pathway[181, 182] potentially through increased production of reactive oxygen species (ROS)[183]. Our findings are consistent with those of Sun *et al.* who found increased expression levels of *ROCK1* but not *ROCK2* and *RhoA* in $PM_{2.5}$-exposed rats compared with rats exposed to filtered air after they were infused with angiotensin II.[182] Along similar lines, evidence in mice indicated that

the RhoA/ROCK pathway plays a fundamental role in $PM_{2.5}$-mediated myocardial remodeling and hypertension.[184]

Immune response pathways

Sex-specific pathways included "defensins" for girls. Most of the genes encoding defensin peptides were down-regulated with increasing long-term $PM_{2.5}$ exposure. Defensins are host defense peptides with antibacterial activity and represent major components of innate immunity. Two subfamilies of defensins, α- and β-defensins, are present in humans: α-defensins are mainly stored in granules of neutrophils and intestinal Paneth cells, while β-defensins are expressed in various epithelial cells. Interestingly, the gene expression of elastase (*ELANE*) and cathepsin G (*CTSG*) [proteases interacting with precursors of α-defensins,[185]] were in the current study also significantly down-regulated and are members of the overrepresented "Urokinase-type plasminogen activator (uPA)" and "uPAR-mediated signaling pathway" (Table 2). Previous studies found a negative association between β-defensin gene expression and residential fly ash, one of the residues generated by oil combustion and being a potential component of $PM_{2.5}$.[186, 187] Decreased levels of antimicrobial peptides, including defensins, may result in higher susceptibility to infections as observed in preterm neonates.[188, 189]

For boys, several immune response pathways involved in both TNF-NF-KB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) and T cell receptor signaling were associated with long-term $PM_{2.5}$ exposure. After adjustment for blood cell count these pathways were no longer significant.

Mitochondrial pathways

Mitochondria, the energy producers of the cells, are particularly sensitive to environmental toxicants due to their lack of DNA repair capacity. Fetuses may adapt their mitochondrial structure and function when the supply of nutrients is limited. Previously, we showed in the ENVIR*ON*AGE birth cohort that placental mitochondrial DNA content[92] and epigenetic modifications[190] in the mitochondrial genome were associated with PM exposure during pregnancy. In line with these findings, we revealed that mitochondrial tricarboxylic acid cycle and respiratory electron chain pathways were significantly linked to short-term gestational $PM_{2.5}$ exposure in boys.

The advantage of our study is that we used a standardized fine-scale exposure assessment enabling us to calculate both short- and long-term exposure on a high resolution scale. Exposure levels in our study were comparable with other European cohort studies. Our study has limitations. First, observational studies do not allow to establish causality. Second, the observed gene expression changes in umbilical cord blood are only indirect evidence of the effects on fetal target tissues such as cardiovascular and nervous tissue. We identified several $PM_{2.5}$-altered genes involved in neural development. A review of 18 studies[191] evaluating comparability of peripheral blood and brain transcriptome data in adults estimated cross-tissue correlation between 0.25 and 0.64 with stronger associations for some subsets of genes and biological processes. Novartis human transcriptomic data[192] showed the following median correlation coefficients between gene expression in whole blood and tissues: immune tissues (R=0.64), central nervous system (R=0.50), peripheral nervous system (R=0.36), heart (R=0.48), and fetal brain (R=0.54). These results support to some extent the use of peripheral blood transcriptome data as surrogate for gene expression in other tissues such as the central nervous system.[191, 192] Maron *et al.*[193] identified fetal biomarkers by comparing gene expression profiles from both maternal and umbilical cord blood in humans. Interestingly, several of the identified transcripts present in both maternal and fetal circulation were identified to be affected by $PM_{2.5}$ exposure in our study both in gene and pathway analysis. This includes immunological and olfactory receptor gene transcripts as well as genes important for development of the nervous system (see table 2 and 3 and Maron *et al.*[193]) Third, our study included 26 (18%) smokers. We adjusted our analyses for maternal smoking status. Although smoking is a major source of personal air pollution exposure, it is unlikely that this biased the current results as we did not find a significant association between maternal smoking and residential air pollution levels. Lastly, the long-term $PM_{2.5}$ concentration in our study ranged from 11.8 to 20.6 μg/m³, with an interquartile range of 2.34 μg/m³. Although this exposure contrast is relatively narrow, previously even smaller contrasts in exposure has been reported in epidemiological studies studying hard clinical endpoints, e.g. the Worcester Heart Attack Study[194] reported a link with acute myocardial infarction for an interquartile range $PM_{2.5}$ exposure contrast of 0.59 μg/m³. Nevertheless, we acknowledge that the small range of $PM_{2.5}$ exposure and the large number of

tests in combination with a small sample size reduces the power of our study. In this regard, we did not apply false discovery rate correction on the individual genes. To improve the reliability of our results, we focused on significant pathways and their genes instead of individual genes. We applied two approaches for the pathway analysis to fully understand the impact of prenatal $PM_{2.5}$ exposure on gene expression: ORA which is based on the *P*-value of individual genes and GSEA which uses the fold change to identify significant pathways. GSEA does not require the use of a significance cut-off at gene level, thereby overcoming the issue of multiple testing. Although the low power of the current study due to the small range of $PM_{2.5}$ exposure in the study region, we believe our study can serve as an exploratory analysis which may inspire further research in this area.

Conclusions

To our knowledge, this is the first study showing a sex-specific link between gestational fine particles and whole genome gene expression in cord blood. The identified transcriptome pathways could provide new molecular insights as to the interaction pattern of early life $PM_{2.5}$ exposure with the biological development of the fetus.

Authors' contributions

TS Nawrot coordinates the ENVIRONAGE birth cohort and designed the current study together with E Winckelmans and K Vrijens. BG Janssen, N Saenen and E Winckelmans constructed the database. E Winckelmans performed the statistical analysis and, with contribution of M Tsamou, the bioinformatical analysis. TM de Kok and J Kleinjans were responsible for the transcriptome analysis. C Vanpoucke and W Lefebvre did the air pollution modelling. E Winckelmans wrote the first draft of the manuscript. All authors were involved in data interpretation and critical revision of the manuscript.

Funding

This research is funded by the European Research Council (ERC-2012-stG310898) and the Flemish Scientific Fund (FWO, 1516112N). Ellen Winckelmans has a PhD. fellowship of Hasselt University (BOF program).

Supplementary material

Table S1. Descriptive characteristics of the ENVIR*ON*AGE birth cohort participants compared to all births in Flanders (Northern part of Belgium) from 2002-2011

Characteristic	ENVIR*ON*AGE subpopulation (n=142)	ENVIR*ON*AGE birth cohort (n=673)*	§Flanders (n=606,877)
Maternal			
Age, yrs	29.4 (24.0-34.0)	29.1 (23.0-35.0)	29.5 (23.5-35.8)
Education			
Low	10.6	11.7	13.1
Medium	35.2	36.0	40.8
High	54.2	52.3	46.1
Parity			
1	50.0	55.9	46.9
2	40.9	34.0	34.7
≥3	9.2	10.1	18.4
Newborn			
Sex			
Boys	45.8	49.8	51.4
Ethnicity			
European-Caucasian	87.3	88.2	87.7
Birth weight, g	3457 (2910, 4045)	3419 (2850, 4004)	3360 (2740, 3965)

Values are percentages or means (10^{th}, 90^{th} percentiles). *from 2010-2014. §[195]

Table S2. Significant differentially expressed genes by long-term $PM_{2.5}$ exposure in cord blood of girls and boys

Gene symbol	Gene name
Up-regulated genes for both sexes	
AP3D1	adaptor-related protein complex 3, delta 1 subunit
C1GALT1	c core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1
CEP131	centrosomal protein 131kDa
FBXW8	F-box and WD repeat domain containing 8
GTF2I	general transcription factor IIi
HECA	hdc homolog, cell cycle regulator
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29)
MAST3	microtubule associated serine/threonine kinase 3
NEAT1	nuclear paraspeckle assembly transcript 1 (non-protein coding)
NOL10	nucleolar protein 10
RBM6	RNA binding motif protein 6
TBC1D31	TBC1 domain family, member 31
TBL1XR1	transducin (beta)-like 1 X-linked receptor 1
TPCN1	two pore segment channel 1
UBXN2A	UBX domain protein 2A
UHMK1	U2AF homology motif (UHM) kinase 1
UPF2	UPF2 regulator of nonsense transcripts homolog (yeast)
UTRN	utrophin
ZFC3H1	zinc finger, C3H1-type containing
Down-regulated genes for both sexes	
ART1	ADP-ribosyltransferase 1
ATG4B	autophagy related 4B, cysteine peptidase
BHLHE23	basic helix-loop-helix family, member e23
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1
CACTIN	cactin, spliceosome C complex subunit
CDC42EP5	CDC42 effector protein (Rho GTPase binding) 5
CHRM1	cholinergic receptor, muscarinic 1
CYGB	cytoglobin
ESR1	estrogen receptor 1
HMG20B	high mobility group 20B
IP6K1	inositol hexakisphosphate kinase 1
KCTD19	potassium channel tetramerization domain containing 19
KRTAP2-4	keratin associated protein 2-4
LINC00320	long intergenic non-protein coding RNA 320
LINC00544	long intergenic non-protein coding RNA 544
MAP1S	microtubule-associated protein 1S
PRR25	proline rich 25
PRR36	proline rich 36
SCGB2B2	secretoglobin, family 2B, member 2
SNORD25	small nucleolar RNA, C/D box 25
SORBS3	sorbin and SH3 domain containing 3
TMEM151B	transmembrane protein 151B

Table S2. Significant differentially expressed genes by long-term $PM_{2.5}$ exposure in cord blood of girls and boys (continued)

Gene symbol	Gene name
Up-regulated genes for boys, down-regulated for girls	
CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7
DNAH10	dynein, axonemal, heavy chain 10
Down-regulated genes for boys, up-regulated for girls	
ALKBH2	alkB homolog 2, alpha-ketoglutarate-dependent dioxygenase
APBA3	amyloid beta (A4) precursor protein-binding, family A, member 3
BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)
BEX2	brain expressed X-linked 2
CD248	CD248 molecule, endosialin
CD6	CD6 molecule
CECR5-AS1	CECR5 antisense RNA 1
CHCHD6	coiled-coil-helix-coiled-coil-helix domain containing 6
CIAO1	cytosolic iron-sulfur assembly component 1
DBH-AS1	DBH antisense RNA 1
EVL	Enah/Vasp-like
FARS2	phenylalanyl-tRNA synthetase 2, mitochondrial
FBXO32	F-box protein 32
IL32	interleukin 32
MCF2L-AS1	MCF2L antisense RNA 1
PCED1B	PC-esterase domain containing 1B
PLAG1	pleiomorphic adenoma gene 1
PRPF39	pre-mRNA processing factor 39
RAB11FIP3	RAB11 family interacting protein 3 (class II)
RASA4	RAS p21 protein activator 4
RPUSD3	RNA pseudouridylate synthase domain containing 3
SEMA4C	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4C
TESPA1	thymocyte expressed, positive selection associated 1
THEMIS	thymocyte selection associated
TLDC1	TBC/LysM-associated domain containing 1
UBQLNL	ubiquilin-like
URB2	URB2 ribosome biogenesis 2 homolog (S. cerevisiae)
WFS1	Wolfram syndrome 1 (wolframin)
ZNF32	zinc finger protein 32
ZNF500	zinc finger protein 500

Table S3. Top ten significant genes in cord blood of newborn boys and girls associated with long-term $PM_{2.5}$ exposure

Sex	Gene symbol	Gene name	FC
Girls (*P*-value <0.0025)			
	CDC42EP5	CDC42 effector protein (Rho GTPase binding) 5	0.85
	ZNF404	zinc finger protein 404	1.60
	SLC25A19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	0.64
	SMYD3	SET and MYND domain containing 3	1.64
	THEM4	thioesterase superfamily member 4	1.52
	ZBTB1	zinc finger and BTB domain containing 1	1.73
	PRR36	proline rich 36	0.64
	SNORD108	small nucleolar RNA, C/D box 108	1.65
	EXOG	endo/exonuclease (5'-3'), endonuclease G-like	1.28
	TSPYL2	TSPY-like 2	1.53
Boys (*P*-value < 0.0001)			
	ZBTB45	zinc finger and BTB domain containing 45	0.80
	SBDS	Shwachman-Bodian-Diamond syndrome	1.70
	C22orf29	chromosome 22 open reading frame 29	0.76
	HSP90AA2P	heat shock protein 90kDa alpha (cytosolic), class A member 2, pseudogene	1.51
	RBM20	RNA binding motif protein 20	0.46
	HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	1.36
	KLHL34	kelch-like family member 34	0.61
	ST13	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	1.44
	SNORA5B	small nucleolar RNA, H/ACA box 5B	0.53
	ROBO2	roundabout guidance receptor 2	0.50

Fold change (FC) calculated for an increase in $PM_{2.5}$ of 5 $\mu g/m^3$.

Table S4. Significant differentially expressed genes by short-term $PM_{2.5}$ exposure in cord blood of girls and boys

Gene symbol	Gene name
Up-regulated genes for both sexes	
ANKRD44	ankyrin repeat domain 44
ARMC8	armadillo repeat containing 8
ATF4	activating transcription factor 4
ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2
C1GALT1	core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1
CHD9	chromodomain helicase DNA binding protein 9
E4F1	E4F transcription factor 1
EDRF1	erythroid differentiation regulatory factor 1
EIF1	eukaryotic translation initiation factor 1
IL6ST	interleukin 6 signal transducer
LARP1	La ribonucleoprotein domain family, member 1
MBD1	methyl-CpG binding domain protein 1
NBPF9	neuroblastoma breakpoint family, member 9
PBRM1	polybromo 1
RPS25	ribosomal protein S25
SRSF1	serine/arginine-rich splicing factor 1
SRSF11	serine/arginine-rich splicing factor 11
UBE2K	ubiquitin-conjugating enzyme E2K
UTRN	Utrophin
VPS13C	vacuolar protein sorting 13 homolog C (S. cerevisiae)
Down-regulated genes for both sexes	
APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2
CACTIN	cactin, spliceosome C complex subunit
CNTNAP1	contactin associated protein 1
CYP4F62P	cytochrome P450, family 4, subfamily F, polypeptide 62, pseudogene
DOCK3	dedicator of cytokinesis 3
DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)
GUF1	GUF1 homolog, GTPase
HAMP	hepcidin antimicrobial peptide
HIC1	hypermethylated in cancer 1
HMG20B	high mobility group 20B
IL2RG	interleukin 2 receptor, gamma
KCTD19	potassium channel tetramerization domain containing 19
KRT7	keratin 7, type II
KRTAP1-3	keratin associated protein 1-3
KRTAP2-4	keratin associated protein 2-4
LINC00320	long intergenic non-protein coding RNA 320
MAP1S	microtubule-associated protein 1S
MTRNR2L10	MT-RNR2-like 10
NACAP1	nascent-polypeptide-associated complex alpha polypeptide pseudogene 1
NUTM2D	NUT family member 2D
PCSK1N	proprotein convertase subtilisin/kexin type 1 inhibitor
PFKL	phosphofructokinase, liver
PIK3CD-AS1	PIK3CD antisense RNA 1

Table S4. Significant differentially expressed genes by short-term $PM_{2.5}$ exposure in cord blood of girls and boys (continued)

Gene symbol	Gene name
PRR25	proline rich 25
PRR36	proline rich 36
SFTPA2	surfactant protein A2
TGFA	transforming growth factor alpha
TMEM151B	transmembrane protein 151B
TOMM20L	translocase of outer mitochondrial membrane 20 homolog (yeast)-like
TSHB	thyroid stimulating hormone, beta
TSPAN11	tetraspanin 11
ZNF205	zinc finger protein 205
ZNF771	zinc finger protein 771
Down-regulated genes for boys, up-regulated for girls	
CECR5-AS1	CECR5 antisense RNA 1
KDM5D	lysine (K)-specific demethylase 5D

Table S5. Top ten significant genes in cord blood of newborn boys and girls associated with short-term $PM_{2.5}$ exposure

Sex	Gene symbol	Gene name	FC
Girls (*P*-value < 0.0026)			
	ASTN2	astrotactin 2	0.84
	THOP1	thimet oligopeptidase 1	1.11
	CDC16	cell division cycle 16	1.22
	MRPS2	mitochondrial ribosomal protein S2	1.12
	ZNF404	zinc finger protein 404	1.30
	TRIM61	tripartite motif containing 61	1.21
	PRP36	proline Rich Protein 36	0.75
	MRPS25	mitochondrial ribosomal protein S25	1.15
	NACAP1	nascent-polypeptide-associated complex alpha polypeptide pseudogene 1	0.72
	SRSF6	serine/arginine-rich splicing factor 6	1.19
Boys (*P*-value < 0.0014)			
	MTRNR2L3	MT-RNR2-like 3	0.75
	AVL9	AVL9 homolog (S. cerevisiase)	1.30
	TNP2	transition protein 2 (during histone to protamine replacement)	0.82
	MTRNR2L7	MT-RNR2-like 7	0.78
	ADAM11	ADAM metallopeptidase domain 11	0.73
	POTEF	POTE ankyrin domain family, member F	1.17
	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1.14
	NRG4	neuregulin 4	0.69
	MTRNR2L10	MT-RNR2-like 10	0.76
	HYALP1	hyaluronoglucosaminidase pseudogene 1	0.63

Fold change (FC) calculated for an increase in $PM_{2.5}$ of 10 $\mu g/m^3$.

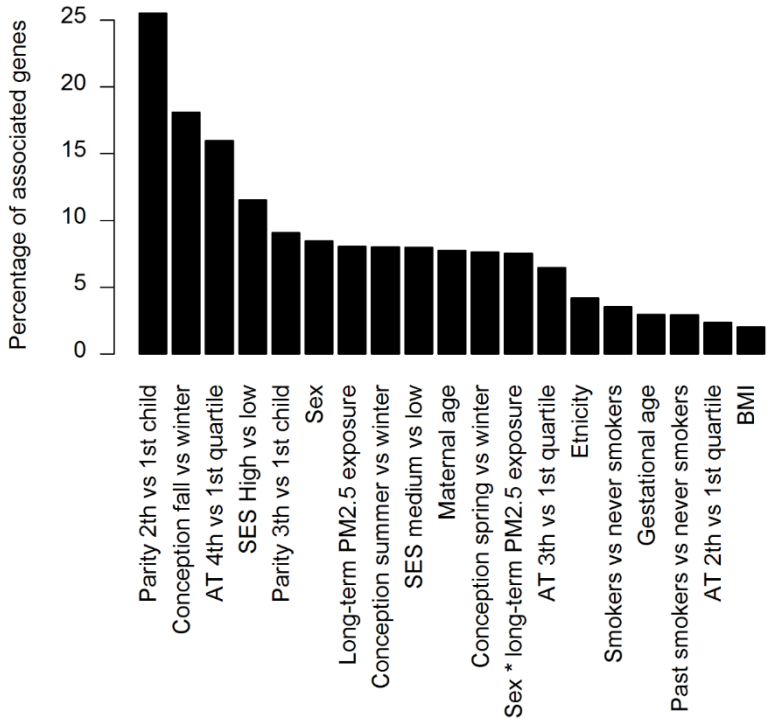

Figure S1. Histogram representing the percentage of genes with *P*-value <0.05 for each variable included in the model.

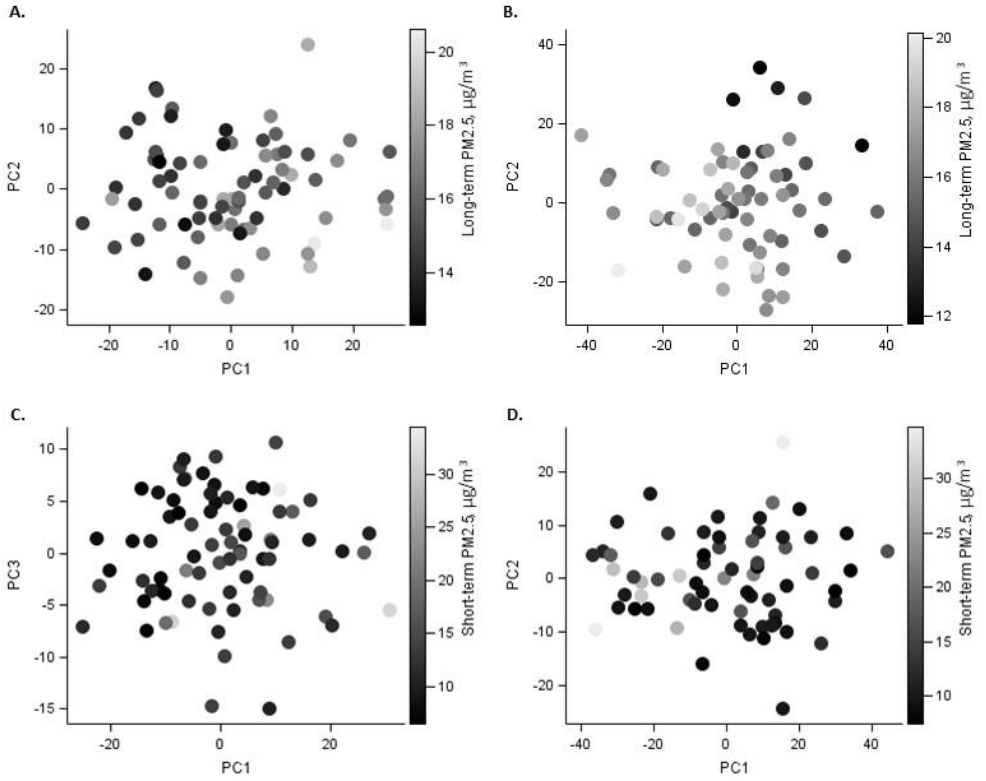

Figure S2. Principal component analysis plot showing the transcriptomic response to long- and short-term $PM_{2.5}$ exposure in *(A, C)* girls and *(B, D)* boys. The plot is based on the $PM_{2.5}$ modulated genes (*P*-value <0.05). A color gradient (dark-light) represent the level of $PM_{2.5}$ exposure (low-high). PC: principal component.

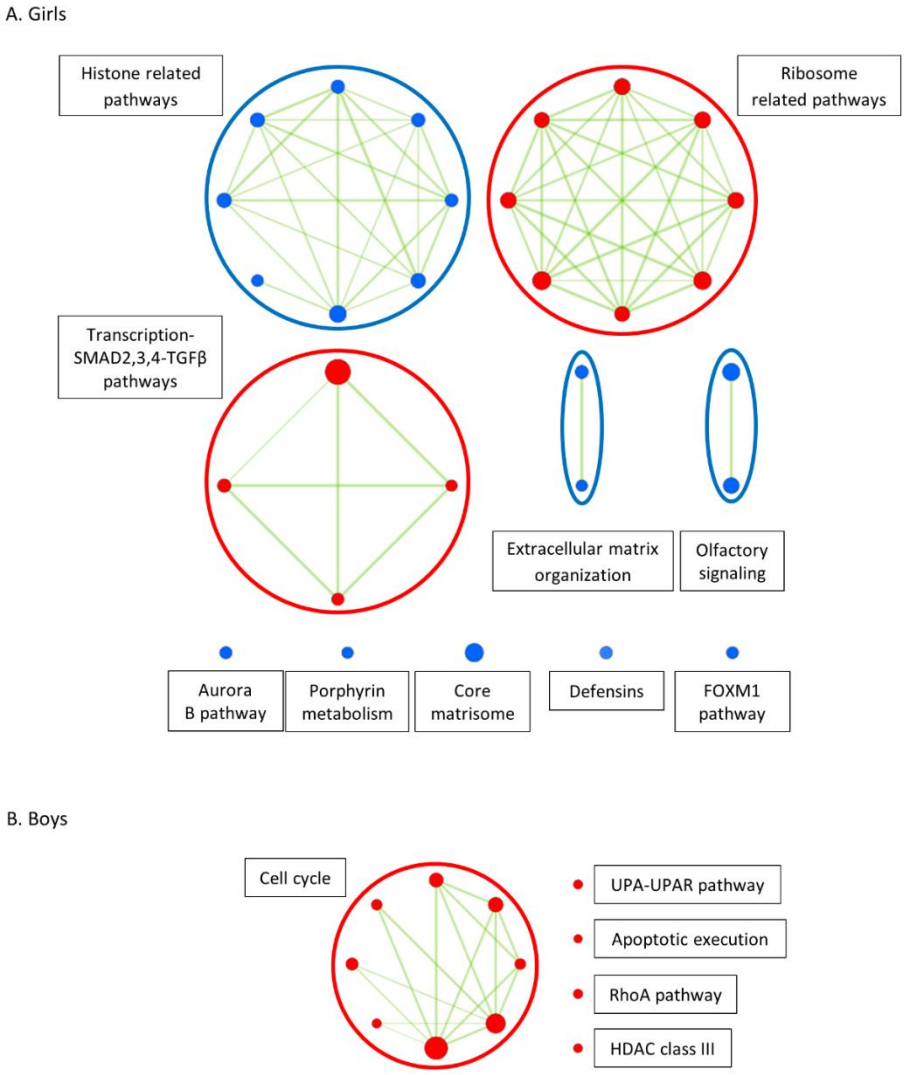

Figure S3. Pathways modulated by long-term $PM_{2.5}$ exposure for girls (*A*) and boys (*B*) resulting from GSEA. The size of the nods represents the size of the pathway. Related pathways are encircled, assigned a label, and connected by green lines, representing common genes between pathways. Up-regulated and down-regulated pathways are given in red and blue, respectively. uPAR: Urokinase-type plasminogen activator (uPA) receptor, HDAC: histone deacetylase, FOXM1: forkhead box M1, RhoA: Ras homolog gene family member A.

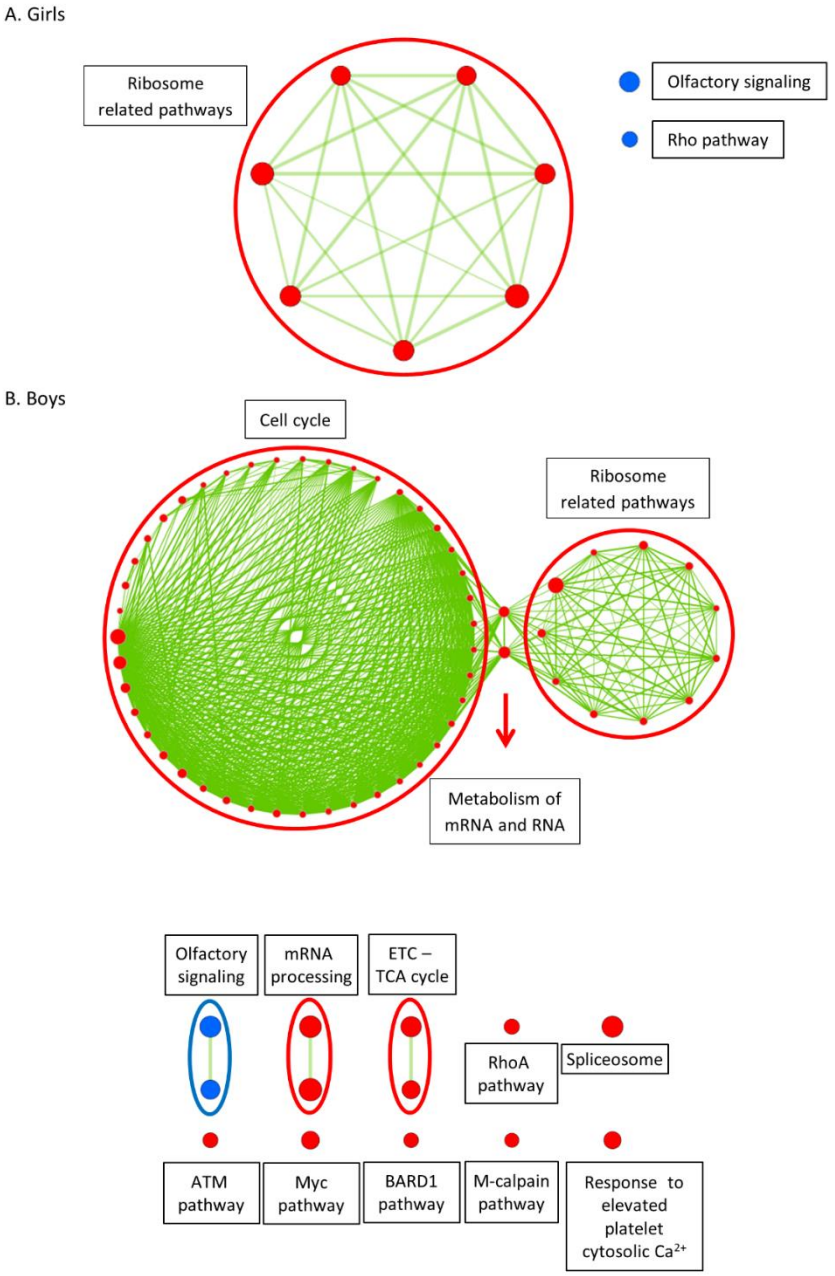

Figure S4. Pathways modulated by short-term $PM_{2.5}$ exposure for girls (A) and boys (B) resulting from GSEA. The size of the nods represents the size of the pathway. Related pathways are encircled, assigned a label, and connected by green lines, representing common genes between pathways. Up-regulated and down-regulated pathways are given in red and blue, respectively. Rho: Ras Homolog gene family, TCA: tricarboxylic acid, ETC: electron transport chain, ATM: Ataxia Telangiectasia Mutated, BARD1: BRCA1 associated RING domain 1. Myc: v-myc avian myelocytomatosis viral oncogene homolog.

Chapter 4

TRANSCRIPTOME-WIDE ANALYSES INDICATE MITOCHONDRIAL RESPONSES TO PARTICULATE AIR POLLUTION EXPOSURE

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Environmental Health 2017; 16:87

Abstract

Background: Due to their lack of repair capacity mitochondria are critical targets for environmental toxicants. We studied genes and pathways reflecting mitochondrial responses to short- and medium-term PM_{10} exposure.

Methods: Whole genome gene expression was measured in peripheral blood of 98 adults (49% women). We performed linear regression analyses stratified by sex and adjusted for individual and temporal characteristics to investigate alterations in gene expression induced by short-term (week before blood sampling) and medium-term (month before blood sampling) PM_{10} exposure. Overrepresentation analyses (ConsensusPathDB) were performed to identify enriched mitochondrial associated pathways and gene ontology sets. 13 Human MitoCarta genes were measured by means of quantitative real-time polymerase chain reaction (qPCR) along with mitochondrial DNA (mtDNA) content in an independent validation cohort (n=169, 55.6% women).

Results: Overrepresentation analyses revealed significant pathways (*P*-value < 0.05) involved in mitochondrial genome maintenance and apoptosis for short-term exposure and to the electron transport chain (ETC) for medium-term exposure in women. For men, medium-term PM_{10} exposure was associated with the Tri Carbonic Acid cycle. In an independent study population, we validated several ETC genes, including *UQCRH* and *COX7C* (*Q*-value <0.05), and some genes crucial for the maintenance of the mitochondrial genome, including *LONP1* (*Q* -value: 0.07) and *POLG* (*Q* -value: 0.04) in women.

Conclusions: In this exploratory study, we identified mitochondrial genes and pathways associated with particulate air pollution indicating upregulation of energy producing pathways as a potential mechanism to compensate for PM-induced mitochondrial damage.

Introduction

Mitochondria are cellular organelles specialized in energy production and produce the majority of intracellular reactive oxygen species (ROS), which are continually generated as toxic by-products by the electron transport chain (ETC). There is a fine balance in ROS signalling maintained by the redox environment. ROS production may be altered as a consequence of exposure to particulate matter (PM). Oxidative stress can occur both when the intracellular and/or intramitochondrial environments are either highly reduced or highly oxidized.[196] Mitochondrial DNA (mtDNA) repairs DNA damage less efficiently compared to nuclear DNA, making it susceptible to ROS and environmental toxicants such as PM.[197] Accumulation of mtDNA damage can cause disturbed mtDNA replication and elimination of damaged mtDNA, and in turn lead to decreased levels of mtDNA.[43, 44] Furthermore, components of the mitochondrial membrane rich in unsaturated fatty acids, such as cardiolipin, are especially sensitive to peroxidation by ROS, resulting in reactive aldehydes which can further damage mitochondrial structures.[43, 198] Increased levels of mtROS or/and accumulation of mitochondrial DNA damage may ultimately lead to programmed cell death.[199] Moreover, oxidative stress and mitochondrial dysfunction are linked with several age-related diseases such as diabetes, cancer, cardiovascular, and neurodegenerative diseases.[45-48]

Here, we explored sex-specific associations of PM exposure on expression of mitochondrial associated genes. Furthermore, a pathway analysis was performed on genome wide transcriptome data to investigate whether mitochondrial pathways are highly affected by air pollution exposure. This hypothesis-generating approach identified sex-specific mitochondrial related genes associated with short- and medium-term PM_{10} exposure that were analysed further in a validation study by means of real-time quantitative PCR (qRT-PCR).

Methods

Study design

As our study aim was to investigate the association of short- and medium-term PM_{10} exposure with mitochondrial-associated transcriptomic responses in

peripheral blood, we performed sex-stratified microarray analyses in a discovery cohort of 98 adults. At gene level, we specifically investigated associations between PM_{10} exposure and expression of 1064 genes listed in the "Human MitoCarta2.0" inventory[200, 201] which are known to encode proteins with mitochondrial localization. Furthermore, we performed pathway analyses on all 15,589 measured transcripts. Based on the gene level and pathway level analyses, we selected 13 MitoCarta genes contributing to top ranked mitochondrial pathways for validation by means of qRT-PCR in an independent validation cohort (n= 169). To substantiate the mitochondrial response to air pollution exposure, we further investigate the link between PM_{10} exposure and mtDNA content in peripheral blood in the validation cohort.

Study population

Discovery cohort

The original study population is part of the first Flemish Environment and Health Survey (FLEHS I) and consisted of 398 individuals from eight different regions of residence in Flanders (Belgium).[202] Inclusion criteria were living in the region of Flanders >5 years, age 50 till 65 years and being able to complete questionnaires in Dutch. Informed consent was obtained from all subjects. Sampling took place between September 2004 and June 2005. Participants donated a blood and urine sample, body height and weight were measured in a standardised way. Demographic data, life style factors and health parameters were provided through an extensive self-assessment questionnaire. A subset of 98 non-smokers was selected for whole genome microarray analysis. The selection procedure was previously described by Vrijens *et al*.[203]

Validation cohort

To validate a selection of MitoCarta genes identified as being associated to PM_{10} exposure in the discovery cohort, we measured whole blood gene expression levels using qRT-PCR in an independent study population of 169 subjects being part of the third Flemish Environment and Health Survey (FLEHS III). Additionally, mtDNA content was determined in peripheral blood of 150 individuals. Inclusion criteria and data collection were similar as for FLESH I. Informed consent was

obtained from all participants. The sampling campaign lasted from May until November 2014.

RNA isolation

Total RNA was extracted from 2.5 ml of whole blood in vacutainers using the Paxgene Blood RNA system (PreAnalytiX, Qiagen, Hilden, Germany), according to the manufacturer's guidelines. A globin reduction assay (GLOBINclear™ Kit by Ambion, Austin, USA) was performed to remove hemoglobin mRNA from samples assessed in microarray analyses. RNA purity was measured spectrophotometrically and RNA integrity was checked using the BioAnalyzer (Agilent, Palo Alto, USA). Labelled samples were assessed for specific activity and dye incorporation.

Microarray preparation, hybridization and preprocessing

From each sample of the discovery cohort, 0.2 µg total RNA was used to synthesize fluorescent cyanine-3-labeled cRNA following the Agilent one-color Quick-Amp labelling protocol (Agilent Technologies). Samples were hybridized on Agilent Human Whole Genome 4x44K microarrays (design ID 014850). Microarrays signals were detected with an Agilent G2505C DNA Microarray Scanner (Agilent Technologies). Raw data were entered in an in-house developed quality control pipeline in R software applying following preprocessing steps: local background correction, omission of controls, flagging of bad quality spots (based on the size of the spot, the number of pixels per spot, the mean vs. median ratio of the pixel intensity, intensity of spot is not above background, and/or saturation of the spot), and spots with too low intensity, $\log_2$-transformation and quantile normalization. Information about the flagging and the R-scripts of the pipeline are available at https://github.com/BiGCAT-UM/arrayQC_Module. Further preprocessing included the omission of probes showing >30% flagged data, merging of replicate probes based on median, and the imputation of missing values using K-nearest neighbor imputation (K=15). If multiple probes represent the same gene, the probe with the largest interquartile range was selected. The final dataset consisted of 15,589 unique Entrez Gene IDs.

Exposure assessment

PM_{10} and $PM_{2.5}$ exposure (µg/m³) concentrations were modelled using a spatial temporal interpolation method (Kriging)[96] for each participants' residential address in combination with a dispersion model. The interpolation method uses land-cover data obtained from satellite images (CORINE land-cover data set) and pollution data collected from a governmental stationary monitoring network. Overall model performance was evaluated by leave-one-out cross-validation including 58 and 34 monitoring points for PM_{10} and $PM_{2.5}$ respectively. Validation statistics of the interpolation tool explained >70% of the temporal variability for hourly and annual PM_{10} and $PM_{2.5}$ averages in the Flemish Region of Belgium.[99] Coupled with a dispersion model (Immission Frequency Distribution Model, IFDM).[97-99] that uses emissions from point sources and line sources, this model chain provides PM values in a dense irregular receptor grid. Previous studies conducted a thorough intercomparison of different models currently in use for regulatory purposes in Europe including IFDM.[204-208] To explore potentially critical exposure windows, we averaged residential one week exposure as a proxy for recent exposure, one month exposure as a proxy for medium-term exposure, two-year exposure as a proxy for long-term exposure. Note that $PM_{2.5}$ exposure estimates were only available for the validation cohort. The Belgian Royal Meteorological Institute provided meteorological data consisting of mean daily air temperature and relative humidity. Apparent temperature was calculated[209, 210] and averaged over the same exposure time window as PM_{10}.

Real-time quantitative PCR (qRT-PCR)

For the validation cohort, total RNA was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). Gene expression was measured in a 10 µl PCR reaction consisting of 2 µL of a 5 ng/µL dilution of cDNA, TaqMan Fast Advanced Master Mix (Life Technologies, Foster City, CA, USA) and PrimeTimeTM assay (Integrated DNA Technologies, Coralville, IA, USA). Samples were analyzed in triplicate with a 7900HT Fast Real-Time PCR system (Life Technologies, Foster City, CA, USA) applying standard cycling conditions. SDS 2.3 provided threshold cycle (C_p) values which were further processed to normalized relative gene expression values with qBase plus

(Biogazelle, Zwijnaarde, Belgium). Replicates were included if the difference in C_p values was <0.5. *HPRT*, *IPO8* and *YWHAZ* were used for data normalization.

DNA extraction and measurement of mtDNA content

For the validation cohort, DNA was isolated from peripheral blood using the QIAmp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's guidelines. The quantity and purity of the extracted DNA were determined using a Nanodrop spectrophotometer (ND-1000; Isogen Life Science B.V., De Meern, the Netherlands). The DNA samples were diluted to 2.4 ng/µL. MtDNA was measured by calculating the relative ratio of two mitochondrial sequences [*MT-ND1* and mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*)] to a single housekeeping nuclear gene (*RPLP0*)] measured using a qPCR assay.[92] qPCR was performed using 2.5 µl extracted DNA and 7.5 µl master mix containing Fast SYBR Green dye 2x (Applied Biosystems, Inc., Foster City, California), forward and reverse primers diluted to 300 nM per well, and RNase-free water. Samples were run in triplicate. Each 384-well plate contained 6 interrun calibrators and 2 no-template controls. 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, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. A melting curve analysis was performed at the end of each run to confirm the absence of nonspecific products. Replicates were included if the difference in C_p values was <0.5. qBase software (Biogazelle, Zwijnaarde, Belgium) was used to normalize C_p values of the two mtDNA sequences relative to the nuclear gene and to correct for run-to-run differences.[211]

Data analysis

Statistical analyses were carried out using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). Continuous data were presented as mean and 10^{th}-90^{th} percentiles and categorical data as percentages and frequencies.

Discovery cohort

For each gene, a multivariable linear regression was fitted to investigate the association between log_2-transformed gene expression levels and PM_{10} exposure estimates (short- and medium-term exposure). Previous epidemiological studies reported that environmental stressors have sex-specific immunological responses, with women being more susceptible to smoking than men.[212, 213] Thereupon, in this study we performed sex-stratified analyses to explore both sex-specific and non-specific PM-induced effects and we adjusted for age, body mass index (BMI), socio-economic status (lower secondary or less, higher secondary, higher education), season (medium [April-May, September-November] or cold [December-March]), time of blood sampling (between 08.20 am and 02.30 pm), apparent temperature and microarray batch number (3 scan dates) to correct for batch effects. Of the 15,589 measured genes, 1064 were "Human MitoCarta2.0" genes[200, 201] which are known to encode proteins with mitochondrial localization. Firstly, because of the specific interest in mitochondria, we performed Human MitoCarta gene-wide association scan, with *P*-values adjusted for multiple testing (for the 1064 genes) by controlling the Benjamini-Hochberg (BH) false discovery rate at 5%. FDR adjusted *P*-values are referred to as *Q*-values. Secondly, we performed pathway analyses. Of the 15,589 genes, genes with unadjusted *P*-value <0.05 were uploaded into the online overrepresentation analysis (ORA) tool ConsensusPathDB (http://consensuspathdb.org/)[164] developed at the Max Planck Institute for Molecular Genetics, to identify processes altered by PM_{10} exposure. Pathways with a *P*-value <0.05 were considered significant.

Validation cohort

Based on the results of the pathway analyses, 7 MitoCarta genes with a *Q*-value < 0.25 (4 in association with short-term exposure and 3 in association with medium-term exposure), contributing to the top 15 ranked pathways/GO terms and with a well-known functional role within mitochondria were selected in women for validation by qRT-PCR. For men, 6 MitoCarta genes (unadjusted *P*-value <0.05) in relation to medium-term exposure and contributing to the top 15 ranked mitochondrial pathways were selected for validation. We examined the association of expression levels measured by qRT-PCR of the 13 selected genes and of mtDNA content with short-, medium-, and long-term PM_{10} and $PM_{2.5}$ exposure. We

adjusted for age, BMI, socio-economic status, smoking, temperature and time as well as season of blood sampling, white blood cell (WBC) count and percentage of neutrophils. Residuals were plotted to check whether significance was driven by outlying gene expression values. Over different time windows, for PM_{10} and $PM_{2.5}$ separately, the BH multiple testing method was applied to correct for the false discover rate (FDR). For the validation cohort, a *Q*-value <0.05 was considered significant.

Results

The characteristics of the discovery and validation cohort are listed in Table 1 for women and men separately. All participants were of European origin. Both cohorts did not differ in the distribution of age and BMI. For both cohorts, age ranged between 50 and 65 years. BMI averaged (range) 26.6 (20.9-38.5) kg/m^2 in the discovery cohort and 25.8 (16.8-39.4) kg/m^2 in the validation cohort. Overall, short- and medium-term PM_{10} exposure estimates were higher in the discovery cohort compared to the validation cohort. In the discovery cohort, more subjects were recruited during the cold period of the year compared to the validation cohort (81.6 vs 39.7%). The discovery cohort consisted only of non-smokers, whilst the validation cohort included 21 (12.4%) smokers. In the validation cohort a higher percentage of participants (53.3%) had a high socio-economic status compared to 28.6% in the discovery cohort.

Gene level analysis

For short- and medium-term exposure, volcano plots of all measured transcripts are presented for both sexes in Figure S1 (Additional file 1). Overall responses to PM_{10} exposure seem to differ between women and men.

Table 2 lists the top 10 Human MitoCarta genes and their corresponding fold changes for an increase in short-term PM_{10} exposure of 10 µg/m^3 for women and men. For women, 8 genes were significantly associated with short-term PM_{10} exposure. The top significant gene for women, *POLG*, encoding the catalytic subunit of the mtDNA polymerase, was downregulated. For men, no significant genes after correction for multiple testing were found. The top ranked gene was

IDI1 required in the mevalonate pathway. None of the 8 significant genes in women were in the top 100 of men.

Table 3 list the top 10 mitochondria-localized genes based on their *P*-value and there corresponding fold changes for an increase in medium-term PM_{10} exposure of 10 μg/m^3 for women and 10 highest ranked genes for men. *ALDH7A1* (*Q*-value: 0.21) and *MRPS15* (*Q*-value: 0.47) were the top ranked genes for women and men respectively.

Table 1. Descriptive characteristics for women and men of the discovery and validation cohort

Characteristics	Discovery cohort (2004-2005)		Validation cohort (2012-2015)	
Personal	**Women (n=50)**	**Men (n=48)**	**Women (n=94)**	**Men (n=75)**
Age, years	57.8 [51.2-63.1]	58.0 [51.5-64.0]	58.1 [52.6-63.2]	58.0 [52.5-63.6]
BMI, kg/m^2	25.8 [22.1-31.1]	27.4 [23.0-31.4]	25.5 [20.4-32.8]	26.1 [21.6-30.9]
WBC, cells/mcL			6965 [5170-9360]	6948 [5200-9270]
Socio-economic status				
Low	28 (56.0)	20 (41.7)	23 (56.0)	14 (18.7)
Medium	7 (14.0)	15 (31.3)	16 (14.0)	26 (34.7)
High	15 (30.0)	13 (27.1)	55 (30.0)	35 (46.7)
Smoking status				
Non-smokers	50 (100.0)	48 (100.0)	80 (85.1)	68 (90.7)
Smokers	-	-	14 (14.9)	7 (9.3)
Season of blood sampling				
Cold (October-March)	40 (80.0)	40 (83.3)	40 (42.6)	27 (36.0)
Warm (April-September)	10 (20.0)	8 (16.7)	54 (57.4)	48 (64.0)
Time of blood sampling				
<12pm	44 (88.0)	41 (85.4)	7 (7.5)	0 (0.0)
12pm-3pm	6 (12.0)	7 (14.6)	25 (26.6)	20 (26.7)
3pm-6pm	0 (0.0)	0 (0.0)	43 (45.7)	32 (42.7)
>8pm	0 (0.0)	0 (0.0)	19 (20.2)	23 (30.7)
Exposure				
Short-term* PM_{10}, μg/m^3	29.3 [18.9-41.3]	30.6 [20.4-41.4]	19.6 [13.0-26.9]	17.8 [12.6-24.0]
Short-term* $PM_{2.5}$, μg/m^3	-	-	12.8 [5.4-26.0]	11.3 [5.8-17.7]
Medium-term§ PM_{10}, μg/m^3	29.7 [24.2-40.5]	31.5 [25.8-40.6]	19.7 [13.8-26.7]	17.5 [13.9-23.8]
Medium-term§ $PM_{2.5}$, μg/m^3	-	-	12.7 [7.4-18.8]	10.8 [7.6-16.9]
Long-term$ PM_{10}, μg/m^3	26.0 [21.4-30.2]	25.7 [21.4-30.1]	24.2 [21.3-27.7]	23.1 [20.8-25.8]
Long-term$ $PM_{2.5}$, μg/m^3	17.8 [15.5-20.5]	17.6 [15.6-20.3]	16.0 [14.9-17.5]	15.5 [14.6-16.5]
Week AT, °C	5.8 [-1.3-11.9]	3.4 [-1.5-11.6]	15.4 [12.7-17.3]	15.2 [12.7-17.3]
Month AT, °C	7.1 [0.6-14.1]	4.7 [0.6-11.2]	15.3 [13.2-16.5]	15.3 [13.2-17.2]

Data are number (%) or mean [10-90th percentile]. AT, apparent temperature.*Week before blood sampling. §Month before blood sampling. $Two-year averages

Table 2. Top 10 most strongly associated Human MitoCarta genes with short-term PM_{10} exposure for women and men

Gene symbol	Gene name	FC (95% CI)	*P*-value	*Q*-value
Women				
POLG	polymerase (DNA) gamma, catalytic subunit	0.83 (0.78, 0.90)	1.31E-05	0.01
MRPL38	mitochondrial ribosomal protein L38	0.83 (0.76, 0.90)	1.08E-04	0.04
MRPL16	mitochondrial ribosomal protein L16	0.83 (0.77, 0.91)	1.46E-04	0.04
OGG1	8-oxoguanine DNA glycosylase	0.81 (0.73, 0.89)	1.48E-04	0.04
ECHS1	enoyl-CoA hydratase, short chain, 1, mitochondrial	0.87 (0.81, 0.93)	1.92E-04	0.04
GTPBP3	GTP binding protein 3 (mitochondrial)	0.82 (0.74, 0.90)	1.99E-04	0.04
ECI2	enoyl-CoA delta isomerase 2	0.84 (0.77, 0.92)	2.86E-04	0.04
ETHE1	ETHE1, persulfide dioxygenase	0.86 (0.79, 0.92)	3.23E-04	0.04
POLRMT	polymerase (RNA) mitochondrial	0.86 (0.80, 0.93)	4.91E-04	0.06
BOLA1	bolA family member 1	0.87 (0.81, 0.93)	5.64E-04	0.06
Men				
IDI1	isopentenyl-diphosphate delta isomerase 1	1.20 (1.08, 1.33)	1.31E-03	0.97
CKMT1A	creatine kinase, mitochondrial 1A	1.30 (1.10, 1.53)	4.09E-03	0.97
PGS1	phosphatidylglycerophosphate synthase 1	1.30 (1.10, 1.54)	4.32E-03	0.97
GNG5	G protein subunit gamma 5	1.12 (1.04, 1.21)	7.37E-03	0.97
TMBIM4	transmembrane BAX inhibitor motif containing 4	1.12 (1.03, 1.22)	1.01E-02	0.97
CHCHD6	coiled-coil-helix-coiled-coil-helix domain containing 6	1.11 (1.03, 1.19)	1.30E-02	0.97
CHCHD8	coiled-coil-helix-coiled-coil-helix domain containing 8	1.17 (1.04, 1.32)	1.44E-02	0.97
COX6A2	cytochrome c oxidase subunit 6A2	1.26 (1.06, 1.50)	1.46E-02	0.97
RPL34	ribosomal protein L34	0.83 (0.72, 0.96)	1.49E-02	0.97
DHRS7B	dehydrogenase/reductase 7B	1.15 (1.03, 1.28)	1.58E-02	0.97

Fold change (FC) calculated for an increase in PM_{10} of 10 $\mu g/m^3$.

Overrepresentation analysis

Sex-specific PM_{10} effects were further explored by overrepresentation analyses (ORA). Table 4 and 5 represent the top 15 significant pathways, with at least 15 measured genes and a total gene size of at most 150 genes, related to, respectively, short- and medium-term PM_{10} exposure for both sexes. For pathways with the same contributing genes, only the most significant pathway is shown. Mitochondrial pathways, containing mainly MitoCarta genes, are marked with an asterisk. Human MitoCarta genes are indicated in bold font.

In women, mitochondrial translation was the top ranked mitochondrial pathway associated with short-term PM_{10} exposure of one week before sampling (Table 4). In addition, PM_{10} exposure was associated with the reaction pathway of busulfan and other DNA damaging agents (*P*-value: 0.004), by deregulating the expression of pro-apoptotic (e.g. *BNIP3*, *LTBR* and *BCL2L1*, isoform Bcl-xS), anti-apoptotic

(e.g. *BCL2L1,* isoform Bcl-xL), DNA repair (e.g. *MLH1)*, and detoxifying genes (e.g. *GSTP1* and *GGT1*). Of these, *BNIP3, BCL2L1* and *MLH1* encode proteins (partially) localized in the mitochondria [200, 201].

Table 3. Top 10 most strongly associated Human MitoCarta genes with medium-term PM_{10} exposure for women and men

Gene symbol	Gene name	FC (95% CI)	*P*-value	*Q*-value
Women				
ALDH7A1	aldehyde dehydrogenase 7 family member A1	0.70 (0.54, 0.90)	2.36E-04	0.21
TIMM17B	translocase of inner mitochondrial membrane 17 homolog B (yeast)	1.05 (0.97, 1.13)	3.90E-04	0.21
GOT2	glutamic-oxaloacetic transaminase 2	0.92 (0.68, 1.24)	8.06E-04	0.23
PDP2	pyruvate dehyrogenase phosphatase catalytic subunit 2	1.04 (0.95, 1.15)	9.34E-04	0.23
GLS	glutaminase	0.79 (0.62, 1.00)	1.40E-03	0.23
FXN	frataxin	0.88 (0.81, 0.97)	1.47E-03	0.23
HINT2	histidine triad nucleotide binding protein 2	0.97 (0.88, 1.06)	2.46E-03	0.23
UQCRH	ubiquinol-cytochrome c reductase hinge protein	0.97 (0.86, 1.09)	2.56E-03	0.23
CPT2	carnitine palmitoyltransferase 2	1.03 (0.94, 1.12)	2.59E-03	0.23
TRUB2	TruB pseudouridine synthase family member 2	0.98 (0.89, 1.07)	3.25E-03	0.23
Men				
MRPS15	mitochondrial ribosomal protein S15	1.23 (1.10, 1.38)	1.14E-03	0.47
CLPB	ClpB homolog, mitochondrial AAA ATPase chaperonin	1.36 (1.15, 1.61)	1.15E-03	0.47
SLC25A29	solute carrier family 25 member 29	1.64 (1.24, 2.16)	1.32E-03	0.47
BCKDK	branched chain ketoacid dehydrogenase kinase	1.24 (1.09, 1.43)	3.37E-03	0.55
STOML1	stomatin-like 1	1.25 (1.08, 1.44)	4.55E-03	0.55
ADCK1	aarF domain containing kinase 1	1.33 (1.10, 1.61)	5.22E-03	0.55
SLC25A40	solute carrier family 25 member 40	0.66 (0.50, 0.87)	6.23E-03	0.55
ALDH7A1	aldehyde dehydrogenase 7 family member A1	1.58 (1.16, 2.16)	6.35E-03	0.55
MDH2	malate dehydrogenase 2	1.29 (1.09, 1.54)	6.67E-03	0.55
ALDH1B1	aldehyde dehydrogenase 1 family member B1	1.36 (1.10, 1.69)	7.33E-03	0.55

Fold change (FC) calculated for an increase in PM_{10} of 10 µg/m^3.

Mitochondrial GO terms associated with short-term PM_{10} exposure included "mitochondrial respiratory chain complex I biogenesis" (*P*-value: 0.001), of which most genes were downregulated, "regulation of mitochondrial membrane permeability" (*P*-value: 0.0007) playing a crucial role in apoptosis and "mitochondrial genome maintenance" (*P*-value: 0.026) including genes important for mitochondrial biogenesis and cardiolipin biosynthesis (e.g. *STOML2*), mtDNA replication (e.g. *POLG*), mitochondria-mediated apoptosis (e.g. *DNAJA3*), and unfolded protein response in the mitochondrial matrix (e.g. *LONP1*). These four Human Mitocarta genes (*Q*-value < 0.25) were selected for validation in an

independent study population. All were downregulated by medium-term PM_{10} exposure, except for *STOML2*.

In men, ORA did not reveal any mitochondrial pathways/GO terms associated with short-term PM_{10} exposure.

The top 15 significant overrepresented pathways associated to medium-term exposure to PM_{10} of one month before sampling are listed for women and men in Table 5. In women, top significant mitochondrial processes altered by medium-term PM_{10} exposure included mitochondrial translation (*P*-value: 0.001) and the respiratory electron transport chain (*P*-value: 0.004). A more detailed overview of the respiratory chain is given in Figure 1. All contributing genes in association to PM_{10} were upregulated except for *ATP5L*, a gene encoding a protein of the ATP synthase complex. *NDUFA13*, *UQCRH*, and *COX7C* (*Q* -value <0.25) were selected for further validation.

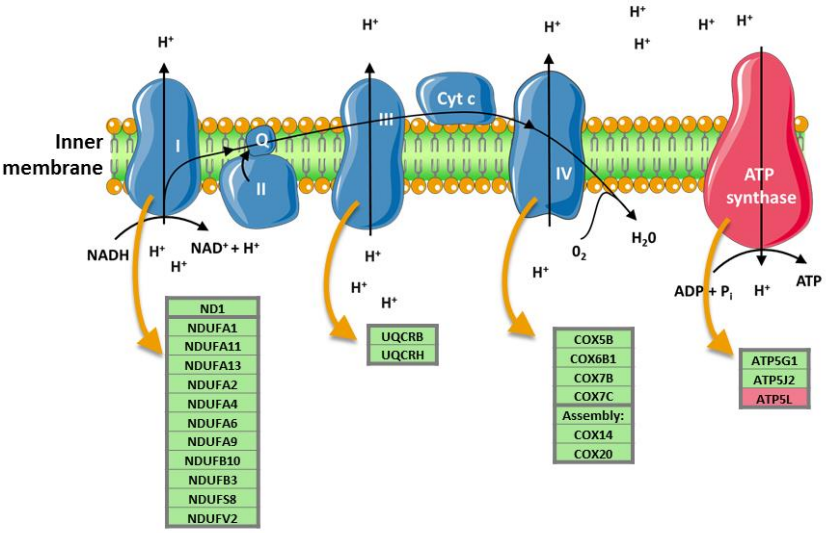

Figure 1. Schematic overview of the mitochondrial respiratory electron transport chain and the genes significantly associated with medium-term PM_{10} exposure per complex in women. Green and red boxes indicate significantly up- and down-regulated genes respectively. Cyt c: cytochrome C.

For men, the tricarboxylic acid (TCA) cycle (*P*-value: 0.0004) was the top mitochondrial pathway associated with medium-term PM_{10} exposure and is represented in Figure 2. Contributing genes in this pathway were all upregulated. *MDH2*, *IDH2*, *PC*, *SUCLA2*, *SDHA*, and *ACO2* (*P*-value < 0.05) were validated in

an independent study population. Other significant pathways including contributing Human MitoCarta genes, were 3-phosphoinositide biosynthesis, IL6-signaling pathway, and histidine metabolism. Phosphoinositide 3-kinases (PI3K) are crucial for various general cellular processes, including cell survival and apoptosis. The IL6-signaling pathway contains the MitoCarta gene *BCL2L1* of which the expression was upregulated in men exposed to relatively high medium-term PM_{10} exposure. Furthermore, the expression of some mitochondria-localized aldehyde dehydrogenases (*ALDH7A1*, *ALDH1B1*), participating in the histidine metabolism pathway, was upregulated by medium-term PM_{10} exposure in men.

ConsensuspathDB analyses revealed overrepresented GO terms regarding mitochondrial functioning consistent with the pathway analysis such as the electron transport chain (*P*-value: 0.008) and mitochondrial translational (*P*-value: 0.001) in women and the TCA cycle (*P*-value: 0.009) in men.

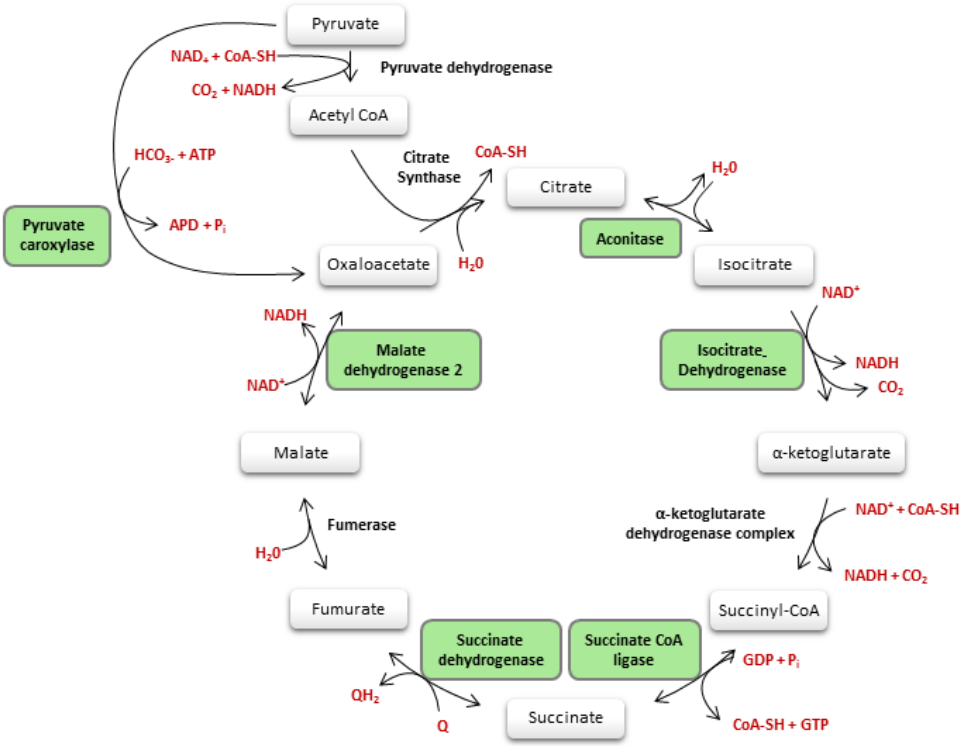

Figure 2. Schematic overview of the TCA cycle. Green boxes indicate significantly up-regulated genes and their corresponding protein in men in association to short-term PM_{10} exposure.

Table 4. Top 15 significant pathways associated with short-term exposure

Pathway	Effective/ total size	# ↓ genes	Contributing genes (#)§	*P*-value
Women				
IL12-mediated signaling events	57/67	33	*TBX21↓;CD247↓;MAPK14↑;CCL4↓;CCR5↓(20)*	1.8E-06
role of mef2d in t-cell apoptosis	28/31	25	*CD247↓; ZAP70↓;FYN↓;LAT↓;CD3E↓;CABIN1↓;PPP3CC↓;LCK↓;PLCG1↓;CAPN2↓; CD3D↓(11)*	1.2E-04
T cell receptor signaling pathway	96/104	52	*CD247↓;PDCD1↓;MAPK14↑;ZAP70↓;FYN↓(22)*	8.0E-04
Ribosome	128/135	100	*RPLP2↓;**MRPL16**↓;RPL36↓;RPL35↓;RPL18↓(27)*	8.6E-04
Natural killer cell mediated cytotoxicity	104/134	50	*CD247↓;HCST↓;ZAP70↓;FYN↓;LAT↓(23)*	1.0E-03
Downstream signaling in naïve CD8+ T cells	52/71	33	*CD247↓;IL2RB↓;EOMES↓;CD8A↓;CD3E↓;PRF1↓;TNFRSF4↓;PTPN7↓;STAT4↓;GZMB↓;BRAF↑;MAPK3↑;CD3D↓;MAPK1↑(14)*	1.4E-03
Formation of a pool of free 40S subunits	94/151	71	*RPLP2↓;RPL36↓;RPL35↓;RPL18↓;RPLP1↓(21)*	1.5E-03
Primary immunodeficiency	32/36	24	*ZAP70↓;ADA↓;RFXAP↓;DCLRE1C↓;CD8A↓;CD3E↓;CD19↓;ICOS↓;LCK↓;CD3D↓(10)*	2.0E-03
TCR signaling in naïve CD8+ T cells	54/58	35	*RASGRP2↓;CD247↓;ZAP70↓;FYN↓;LAT↓;CARD11↓;CD8A↓;CBL↑;RASGRP1↓;CD3E↓; PRF1↓;LCK↓;PLCG1↓;CD3D↓(14)*	2.0E-03
Mitochondrial translation (elongation)*	84/85	72	***MRPL38**↓;**MRPL16**↓;**MRPS9**↓;**MRPL4**↓;**MRPS26**↓(19)*	2.1E-03
NF-κB signaling pathway	86/91	43	*PARP1↓;TRAF2↓;ZAP70↓;CCL4↓;TRAF5↓(19)*	2.8E-03
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	64/132	41	*CD247↓;HCST↓;ICAM4↑;ITGB7↓;CD8A↓;CD96↓;KIR2DL2↓;CD3E↓;CD19↓;KIR3DL1↓; KLRD1↓;KIR3DL2↓;ITGB1↓;ITGA4↓;CD3D↓(15)*	4.2E-03
Busulfan Pathway, Pharmacodynamics	30/36	16	***BNIP3**↓;CHEK2↓;**BCL2L1**↑;**MLH1**↓;FMO5↑;LTBR↑;GSTP1↓;GGT1↑;MPG↓(9)*	4.5E-03
Cell cycle	116/124	63	*ZBTB17↓;ANAPC1↓;CDK4↓;MCM7↓;CHEK2↓(23)*	4.6E-03
Downregulation of SMAD2/3:SMAD4 transcriptional activity	20/21	10	*PARP1↓;NCOR2↓;RPS27A↓;SMAD3↓;UBB↑;PPM1A↑;NEDD4L↑(7)*	4.6E-03
Men				
Meiotic recombination	53/64	3	*HIST1H3C↑;HIST1H2B↑;HIST2H3A↑;HIST1H2B↑;HIST3H2B↑(22)*	1.3E-12
Cytokine Signaling in Immune system	156/198	49	*STAT2↑;IRS2↑;CSH1↑;PELI1↑;EIF4E3↑(24)*	1.7E-04
Osteoclast differentiation	120/131	25	*CYBA↑;FCGR3B↑;FOSL2↑;GRB2↑;IFNAR1↑(20)*	2.0E-04
Phagosome	135/155	35	*ATP6V1E1↑;CD14↑;CYBA↑;CLEC4M↑;FCAR↑(21)*	3.7E-04
GMCSF-mediated signaling events	36/41	10	*FOS↑;PRKACA↑;STAT5B↑;OSM↑;GRB2↑;MAP2K1↑;LYN↑;STAT5A↑;MAPK3↑(9)*	6.0E-04
Legionellosis	51/55	13	*CASP1↑;CD14↑;CXCL3↑;HBS1L↑;HSPA1A↑;IL1B↑;MYD88↑;RAB1A↑;TLR2↑;TLR4↑; VCP↑(11)*	6.1E-04
Tuberculosis	151/179	46	*CEBPB↑;CTSD↑;CD14↑;RHOA↑;CLEC4M↑(22)*	6.9E-04

Table 4. Top 15 significant pathways associated with short-term exposure (continued)

Pathway	Effective/ total size	# ↓ genes	Contributing genes (#)[§]	*P*-value
Growth hormone receptor signaling	17/20	1	*IRS2*↑;*CSH1*↑;*SOCS3*↑;*STAT5B*↑;*LYN*↑;*STAT5A*↑*(6)*	6.9E-04
Oncostatin_M	37/40	9	*CEBPB*↑;*FOS*↑;*OSMR*↑;*SOCS3*↑;*JUNB*↑;*STAT5B*↑;*OSM*↑;*GRB2*↑;*MAPK3*↑*(9)*	7.5E-04
IL3 Signaling Pathway	47/40	13	*MAP2K1*↑; *LYN*↑; *GRB2*↑; *PRKACA*↑; *HCK*↑; *MAPK3*↑; *STAT5B*↑;*VAV1*↑;*FOS*↑;*STAT5A*↑*(10)*	1.2E-03
Endogenous Toll-like receptor signaling	25/27	5	*CD14*↑;*TLR4*↑;*RHOA*↑;*TLR2*↑;*TLR1*↑;*MYD88*↑;*TLR6*↑*(7)*	1.2E-03
Salmonella infection	73/86	20	*MYD88*↑;*ACTB*↑;*RAB7*↑;*FOS*↑;*CASP1*↑;*CXCL3*↑;*ARPC4*↑;*IFNGR2*↑;*CD14*↑;*MYH14*↑;*TLR4*↑ ;*MAPK3*↑;*IL1B*↑*(13)*	1.4E-03
Kit receptor signaling pathway	57/59	11	*MAP2K1*↑;*LYN*↑;*GRB2*↑;*GRB7*↑;*JUNB*↑;*MAPK3*↑;*STAT5A*↑;*STAT5B*↑;*RPS6KA1*↑; *VAV1*↑;*FOS*↑*(11)*	1.6E-03
Toll-like receptor signaling pathway	84/102	18	*IL1B*↑;*MAPK3*↑;*CD14*↑;*TLR6*↑;*MAP2K1*↑;*IRF7*↑;*TLR1*↑;*IKBKE*↑;*TLR2*↑;*IFNAR1*↑;*TLR4*↑; *FADD*↑;*FOS*↑;*MYD88*↑*(14)*	1.8E-03
Cytoplasmic Ribosomal Proteins	85/88	73	***RPS18*↓;***RPL27*↓;*RPL27A*↓;***RPL10A*↓;***RPL19*↓;*RPL18*↓;*RPLP1*↓;***RPL34*↓;***RPS8*↓;*RPL13A*↓; *RPS6KA1*↑;*RPL11*↓;*RPS29*↓;*RPS27*↓*(14)*	2.0E-03

↓ Number of down-regulated genes. [§]If more than 15 contributing genes only the top 5 is given. Mitochondrial pathways are marked with an asterisk and MitoCarta genes are indicated in bold type. IL: Interleukin; MEF2D: myocyte enhancer factor 2D; SMAD2,3,4: SMAD family member 2,3,4; GMCSF: Granulocyte-macrophage colony-stimulating factor

Table 5. Top 15 significant pathways associated with medium-term exposure

Pathway	Effective/ total size	# ↓ genes	Contributing genes (#)[§]	*P*-value
Women				
RNA Polymerase I Chain Elongation	79/98	25	*HIST2H2A↑;HIST1H2A↑;HIST1H4E↑;HIST1H2A↑;POLR1E↓(24)*	1.7E-04
Meiosis	63/77	20	*HIST2H2A↑;HIST1H2A↑;HIST1H4E↑;HIST1H2A↑; HIST1H4I↑(20)*	3.0E-04
RMTs methylate histone arginines	64/74	23	*HIST2H2A↑;RBBP7↓;HIST1H2A↑;HIST1H2A↑; HIST1H4E↑(20)*	3.8E-04
RNA Polymerase I Transcription	100/121	35	*HIST2H2A↑;RBBP7↓;HIST1H2A↑;HIST1H4E↑; HIST1H2A↑(27)*	5.7E-04
Mitochondrial translation (termination)*	84/85	25	***MRPL50↓;MRPL22↑;MRPL30↑;MRPL47↑;MRPL54↑****(23)*	1.1E-03
phospholipids as signalling intermediaries	30/36	12	*EDG1↓;MAP2K1↓;ITGAV↓;SOS1↑;SRC↑;PDGFA↑;PIK3CA↓; ASAH1↑;PDPK1↑;HRAS↑;ADCY1↓(11)*	1.9E-03
Meiotic recombination	53/64	15	*HIST2H2A↑;HIST1H2A↑;HIST1H4E↑;HIST1H2A↑;HIST1H4I↑(16)*	2.1E-03
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins.*	99/113	23	***UQCRH↑;ATP5L↓;NDUFA13↑;COX7C↑;UCP3↑****(25)*	2.5E-03
Meiotic synapsis	40/48	8	*HIST1H4E↑;HIST1H4I↑;HIST1H2A↑;HIST4H4↑;RAD21↓;HIST1H4H↑;ATR↓; HIST2H4B↑;HIST1H4J↑;BRCA1↑;HIST1H4K↑;REC8L1↑; HIST1H2B↑(13)*	2.6E-03
Sirtuin 1 negatively regulates rRNA Expression	59/76	18	*HIST1H4H↑;HIST2H2A↑;HIST1H4I↑;HIST1H2A↑; HIST1H2A↑(17)*	2.7E-03
Condensation of Prophase Chromosomes	64/77	17	*HIST2H2A↑;HIST1H2A↑;HIST1H4E↑;HIST1H2A↑; HIST1H4I↑(18)*	2.8E-03
NoRC negatively regulates rRNA expression	95/116	34	*HIST2H2A↑;HIST1H2A↑;HIST1H4E↑;HIST1H2A↑; POLR1E↓(24)*	3.0E-03
RNA Polymerase I, RNA Polymerase III, and Mitochondrial Transcription	133/159	48	*HIST2H2A↑;RBBP7↓;HIST1H2A↑;HIST1H4E↑;HIST1H2A↑(31)*	3.2E-03
Platelet Aggregation (Plug Formation)	28/38	9	*GP1BA↑;SOS1↑;SRC↑;VWF↑;RASGRP2↓;GP9↑;ITGA2B↑;PDPK1↑;TLN1↑;RAP1A↓(10)*	3.7E-03
Respiratory electron transport*	81/92	17	***UQCRH↑;NDUFA13↑;COX7C↑;FAM36A↑;NDUFA6↑****(21)*	3.8E-03
Men				
3-phosphoinositide biosynthesis	25/29	10	*C17orf38↑;PIK4CB↑;PIK3R1↑;PIP5K3↓;**PIK4CA↑;**CDIPT↑;PIP5K2B↑;PIK3R2↑(8)*	2.0E-04
superpathway of inositol phosphate compounds	62/71	31	*C17orf38↑;PIK4CB↑;PIK3R1↑;TMEM55A↓;PIP5K3↓;IHPK2↑;SKIP↑;**PIK4CA↑;**CDIPT↑; PIP5K2B↑;PIK3R2↑;OCRL↓;HISPPD1↓(13)*	2.8E-04
TCA cycle*	27/34	7	***MDH2↑;IDH2↑;IDH3G↑;SUCLA2↑;SDHA↑;CLYBL↑;PCK2↑;ACO2↑****(8)*	3.7E-04
Citrate cycle (TCA cycle)*	28/30	9	***MDH2↑;IDH2↑;PC↑;IDH3G↑;SUCLA2↑;SDHA↑;PCK2↑;ACO2↑(8)***	4.8E-04
PI Metabolism	47/53	19	*C17orf38↑;PIK4CB↑;PIK3R1↑;PIP5K3↓;SKIP↑;PIK4CA↑;PIP5K2B↑;ARF1↑;PIK3R2↑; OCRL↓(10)*	1.3E-03
IRS-related events triggered by IGF1R	74/93	26	*GBL↑;MAP2K2↑;FGFR4↑;IGF2↑;STK11↑;PIK3R1↑;DOK1↑;FGF4↑;AKT2↑;TYK2↑; PIK3R2↑;TLR9↓;SOS1↓(13)*	1.7E-03
Warburg Effect*	43/45	15	***PC↑;IDH3G↑;GAPDH↑;****SLC1A5↑;**SDHA↑;**ENO1↑;PKM2↑;**ACO2↑;**PGD↑(9)*	2.5E-03
Synthesis of PIPs at the Golgi membrane	15/20	6	*PIK4CB↑;PIP5K3↓;PIK4CA↑;ARF1↑;OCRL↓(5)*	2.7E-03

Table 5. Top 15 significant pathways associated with medium-term exposure (continued)

Pathway	Effective/ total size	# ↓ genes	Contributing genes (#)[§]	*P*-value
superpathway of conversion of glucose to acetyl CoA and entry into the TCA cycle	44/52	13	*MDH2*↑;*GCK*↑;*IDH3G*↑;*GAPDH*↑;*SUCLA2*↑;*SDHA*↑;*ENO1*↑;*PKM2*↑;*ACO2*↑*(9)*	2.9E-03
Histidine metabolism	23/36	7	***ALDH7A1***↑;***ALDH1B1***↑;*SLC38A5*↑;*SLC1A4*↑;*SLC1A5*↑; *SLC38A3*↑*(6)*	4.1E-03
Ghrelin	31/44	7	*PIK3R1*↑;*DOK1*↑;*AP2M1*↑;*NOS3*↑;*GNAI2*↑;*PRKCE*↓; *RICTOR*↓*(7)*	4.7E-03
IL6 signaling pathway	39/43	9	*MAP2K2*↑;*PIK3R1*↑;*HCK*↑;*NR2F6*↑;*TYK2*↑;*PIK3R2*↑;***BCL2L1***↑;*SOS1*↓*(7)*	4.8E-03
Cell-Cell communication	95/130	29	*PIK3R1*↑;*ACTN4*↑;*PARD6G*↑;*ACTN3*↑;*PAK1*↑;*SIRPG*↑;*KIRREL2*↑;*DSCAM*↑;*CDH24*↑; *PIK3R2*↑;*CDH3*↑;*CLDN23*↑;*CLDN3*↑;*FLNC*↑*(14)*	6.0E-03
Regulation of toll-like receptor signaling pathway	116/142	38	*GBL*↑;*MAP2K2*↑;*PIK3R1*↑;*IKBKE*↑;*SQSTM1*↑*(16)*	6.6E-03
Downstream signaling of activated FGFR	127/155	46	*CDKN1A*↑;*GBL*↑;*MAP2K2*↑;*FGFR4*↑;*PIK3R1*↑*(17)*	7.0E-03

↓ Number of down-regulated genes. [§] If more than 15 contributing genes only the top 5 is given. Mitochondrial pathways are marked with an asterisk and MitoCarta genes are indicated in bold type. RMTs: arginine methyltransferases; IGF1R: insulin-like growth factor 1; FGFR: fibroblast growth factor receptors.

Validation

For the validation cohort, both PM_{10} and $PM_{2.5}$ estimates were available. Results on long-term PM_{10} exposure in the discovery cohort were published in a previous paper[203]. However, analysis of short- and medium-term exposure with microarray data in the discovery cohort and qPCR validation of MitoCarta genes in an independent cohort is novel. Table 6 present the fold changes (95% CI) and *P*-values for the linear association between the 7 MitoCarta genes selected for women in the discovery cohort and short-, medium-, and long-term PM_{10} and $PM_{2.5}$ exposure in women and men of the validation cohort. For women, several of the selected genes were associated with medium- and/or long-term PM exposure. Of the genes contributing to mitochondrial genome maintenance, expression levels of *POLG* and *LONP1* were negatively associated with long-term $PM_{2.5}$ exposure (*Q*-value: 0.04 and 0.07 respectively) and *DNAJA3* and *LONP1* were downregulated by medium-term $PM_{2.5}$ exposure (*Q*-value: 0.05 and 0.07 respectively). ETC genes were upregulated by PM_{10} and $PM_{2.5}$ for all time windows, however only significantly for the association between long-term $PM_{2.5}$ exposure and *COX7C* and *UQCRH* gene expression (Q-value < 0.05). For men, none of the selected TCA contributing genes could be validated. However, consistent with the observations in women, *LONP1* was negatively associated and *UQCRH* and *NDUFA13* were positively associated with long-term PM_{10} and $PM_{2.5}$ exposure in men of the validation cohort. However, after FDR correction associations in men were not significant.

Mitochondrial DNA content

Mitochondrial DNA content was negatively associated with short-, medium-, and long-term PM_{10} and $PM_{2.5}$ exposure in women (Table 7). For men, mtDNA content was negatively associated with short-term PM_{10} and $PM_{2.5}$ exposure whilst medium-term PM_{10} and $PM_{2.5}$ exposure revealed a trend towards significance. No significant associations were observed between mtDNA content and the expression of the 13 selected Human MitoCarta genes.

Table 6: Association between expression levels of the selected genes and PM exposure in women (n=94) and men (n=75) of the validation cohort

Sex	Gene symbol	FC (95%CI)	P-val	FC (95%CI)	P-val	FC (95%CI)	P-val	FC (95%CI)	P-val	FC (95%CI)	P-val	FC (95%CI)	P-val
		Short-term PM_{10}		Short-term $PM_{2.5}$		Medium-term PM_{10}		Medium-term $PM_{2.5}$		Long-term PM_{10}		Long-term $PM_{2.5}$	
Women	**Mt genome**												
	POLG	0.76 (0.57,1.00)	0.06	0.75 (0.57,1.00)	0.05	0.65 (0.39,1.10)	0.11	0.57 (0.32,1.01)	0.06	0.90 (0.78,1.03)	0.12	0.62 (0.45,0.86)	0.005$$
	STOML2	1.13 (0.92,1.37)	0.25	1.11 (0.91,1.35)	0.32	0.95 (0.68,1.34)	0.78	0.95 (0.65,1.39)	0.80	1.03 (0.94,1.13)	0.57	1.21 (0.97,1.51)	0.09
	DNAJA3	0.92 (0.81,1.05)	0.23	0.90 (0.79,1.03)	0.13	0.79 (0.63,0.99)	0.04	0.72 (0.56,0.93)	0.01$	0.96 (0.90,1.02)	0.22	0.88 (0.76,1.03)	0.11
	LONP1	0.84 (0.66,1.09)	0.20	0.84 (0.65,1.08)	0.19	0.64 (0.40,1.00)	0.05	0.55 (0.34,0.90)	0.02$	0.91 (0.80,1.03)	0.15	0.70 (0.52,0.93)	0.02$
	ETC												
	COX7C	1.22 (0.85,1.77)	0.28	1.19 (0.83,1.72)	0.35	1.50 (0.78,2.86)	0.22	1.56 (0.76,3.22)	0.23	1.19 (1.01,1.41)	0.04	1.82 (1.23,2.70)	0.004$$
	UQCRH	1.32 (0.90,1.93)	0.16	1.29 (0.88,1.89)	0.19	1.29 (0.66,2.49)	0.46	1.42 (0.69,2.94)	0.35	1.14 (0.96,1.37)	0.15	1.81 (1.20,2.71)	0.006$$
	NDUFA13	1.15 (0.89,1.50)	0.30	1.12 (0.87,1.46)	0.38	1.01 (0.65,1.59)	0.95	1.03 (0.63,1.69)	0.90	1.05 (0.93,1.19)	0.45	1.37 (1.03,1.81)	0.03
Men	**Mt genome**												
	POLG	0.94 (0.86, 1.03)	0.18 [a]	0.95 (0.87, 1.04)	0.33 [a]	0.90 (0.77, 1.04)	0.16 [a]	0.90 (0.77, 1.05)	0.17 [a]	0.96 (0.92, 1.01)	0.09 [a]	0.91 (0.82, 1.01)	0.07 [a]
	STOML2	0.96 (0.86, 1.08)	0.52	0.98 (0.88, 1.1)	0.78	0.88 (0.73, 1.06)	0.17	0.88 (0.72, 1.07)	0.19	1.01 (0.96, 1.06)	0.76	0.99 (0.87, 1.12)	0.88
	DNAJA3	1.01 (0.92, 1.10)	0.85	1.02 (0.94,1.11)	0.61	1.08 (0.94, 1.24)	0.29	1.08 (0.94, 1.26)	0.29	1.03 (0.99, 1.07)	0.22	1.05 (0.96, 1.16)	0.27
	LONP1	0.90 (0.81, 1.11)	0.52 [b]	0.98 (0.84, 1.14)	0.82 [b]	0.86 (0.67, 1.11)	0.25 [b]	0.89 (0.68, 1.15)	0.37 [b]	0.93 (0.87, 1.00)	0.04 [b]	0.80 (0.68, 0.94)	0.01 [b]
	ETC												
	COX7C	1.09 (0.85, 1.40)	0.49	1.11 (0.88, 1.41)	0.38	1.17 (0.78, 1.75)	0.46	1.23 (0.81, 1.88)	0.34	1.08 (0.96, 1.21)	0.190	1.23 (0.94, 1.60)	0.14
	UQCRH	1.14 (0.92, 1.41)	0.25	1.16 (0.94, 1.44)	0.16	1.39 (0.97, 1.98)	0.07	1.41 (0.97, 2.04)	0.07	1.12 (1.01, 1.24)	0.04	1.32 (1.04, 1.69)	0.03
	NDUFA13	1.03 (0.93, 1.15)	0.58	1.05 (0.95, 1.17)	0.36	1.14 (0.96, 1.35)	0.14	1.13 (0.95, 1.36)	0.18	1.05 (1.00, 1.10)	0.04	1.13 (1.01, 1.27)	0.04

FC: Fold changes for an increase in PM of 10 µg/m^3 (short- and medium-term) and 2 µg/m^3 (long-term). Adjusted for age, BMI, smoking status, educational level, and time of blood sampling, temperature, WBC count, and percentage of neutrophils. Mt: mitochondrial. [a] 3 outliers with relatively low *POLG* expression removed. [b] 1 outlier with relatively high *LONP1* expression removed. Results including the outlier were similar (long-term $PM_{2.5}$ p-val=0.007). FDR-adjusted p-values < 0.05$$ and <0.10$

Table 7: Association between mtDNA content and PM exposure

	Men (n=67)		Women (n=83)	
Time window	**FC (95% CI)**	**P-val**	**FC (95% CI)**	***P*-value**
Short-term PM_{10}	0.80 (0.67, 0.96)	0.02	0.82 (0.69, 0.97)	0.02
Short-term $PM_{2.5}$	0.82 (0.69, 0.98)	0.03	0.83 (0.7, 0.98)	0.04
Medium-term PM_{10}	0.77 (0.57, 1.04)	0.09	0.74 (0.55, 0.99)	0.05
Medium-term $PM_{2.5}$	0.75 (0.55, 1.03)	0.08	0.73 (0.53, 1.01)	0.06
Long-term PM_{10}	0.95 (0.87, 1.04)	0.26	0.9 (0.83, 0.97)	0.007
Long-term $PM_{2.5}$	0.88 (0.72, 1.08)	0.22	0.76 (0.64, 0.91)	0.004

Fold changes (FC) for an increase in PM of 10 $\mu g/m^3$ (short- and medium-term) and 2 $\mu g/m^3$ (long-term). Adjusted for age, BMI, smoking status, educational level, and time of blood sampling, temperature and WBC count and percentage of neutrophils.

Discussion

The current study identified several mitochondrial-related genes and pathways significantly associated with fine particle exposure at different exposure time windows: short- (one week before the blood sampling) and medium-term (one month before the blood sampling) PM_{10} exposure. For women, PM exposure affected, among others, pathways contributing to mitochondrial genome maintenance (short-term), electron transport chains (short-, medium-term) and mitochondrial translation (short- and medium-term). For men, the TCA cycle was positively associated with medium-term PM_{10} exposure. Furthermore, we were able to validate a selection of mitochondrial-linked genes in an independent study population.

Transcriptome-wide long-term (two-year averages) results of the discovery cohort were described in a previous paper identifying potential gene expression biomarkers of PM exposure.[203] In line with the results for medium-term exposure, the electron transport chain was significantly associated with long-term PM_{10} and $PM_{2.5}$ exposure in women.

For women, we selected three genes (*COX7C, UQCRH,* and *NDUFA13*), associated with medium-term PM_{10} exposure in the discovery cohort, encoding proteins contributing to the electron transport chain complexes and four genes (*POLG*, *STOML2, DNAJA3,* and *LONP1*) significantly associated with short-term PM_{10} exposure in the discovery cohort, of which their corresponding proteins play a role

in mitochondrial genome maintenance. For all genes, the direction of association, by PM exposure during the significant time window of the discovery cohort, were replicated in the validation cohort by qPCR. We validated *POLG*, *LONP1*, *COX7C* and *UQCRH* in relation to long-term PM exposure and *DNAJA3* and *LONP1*, for medium-term exposure. For men, none of the selected TCA contributing genes could be validated. However, consistent with the results for women, *UQCRH,* and *NDUFA13* were upregulated and *LONP1* was down-regulated by long-term PM exposure in the validation cohort. Possibly, we could validate most genes only for long-term exposure because this exposure estimate is independent of season of blood sampling, which partly differs between the two study cohorts. For the validation cohort more pronounced effects were observed for $PM_{2.5}$ compared to PM_{10}, however, for each time window the correlation coefficient between $PM_{2.5}$ and PM_{10} was > 0.85.

In accordance with our study results, Hoffmann and colleagues observed significantly increased ETC protein levels (Complex II, III, V) in the human bronchial epithelial cell line BEAS-2B in response to cigarette smoke exposure for 6 months.[214] The upregulated expression of ETC genes (Figure 1), as observed in the current study, and ETC proteins due to environmental toxicant exposure may indicate increased energy demand required to eliminate damage to cellular components.

To further explore mitochondrial responses to PM exposure, we investigated mtDNA content in the validation cohort. In line with the results at the gene expression level (downregulation of genes important for mitochondrial genome replication such as *POLG*, *POLG2*, and *POLRMT*), mtDNA content, measured in the validation cohort, was decreased among both women and men exposed to relatively high PM levels for short-, medium- and long-term. To date, several studies reported a deregulation of mtDNA content in response to environmental factors.[43, 92, 215-218] However, the direction of effect is not consistent over these studies. Differences in exposure levels and population characteristics often make it difficult to compare study findings. The findings of the current study are in line with previous evidence reporting the ability of ultrafine particles to induce oxidative stress and mitochondrial damage,[219] and the selective elimination of damaged mtDNA in order to help maintain mtDNA integrity.[44] Cline hypothesised that poly aromatic hydrocarbons, toxic components of PM, can block mtDNA

polymerase and topoisomerase activity and in turn reduce mtDNA replication.[43] In this study, we observe a reduction at the level of gene expression of *POLG*, *POLG2* and *POLRMT* which may further explain the decreased mtDNA content in individuals exposed to relatively high PM exposure. Presumably, decreased mtDNA content and mitochondrial damage stimulate transcription factors regulating the expression of electron transport genes in order to provide the required energy to eliminate cellular damage. Mitochondrial dysfunction can augment ROS production which may in turn activate the mitochondrial apoptotic pathway. The altered expression of pro- and anti-apoptotic genes (such as gene members of the BCL-2 family and caspases) in response to short-term PM_{10} exposure in women and of members of the PI3K/AKT (busulfan) pathway, which delivers an anti-apoptotic signal, in men further supports the theory that PM-induced formation of ROS can influence mitochondrial function and regulate cell fate.[220] Figure 3 shows a schematic overview of the potential effect of PM on mitochondria.

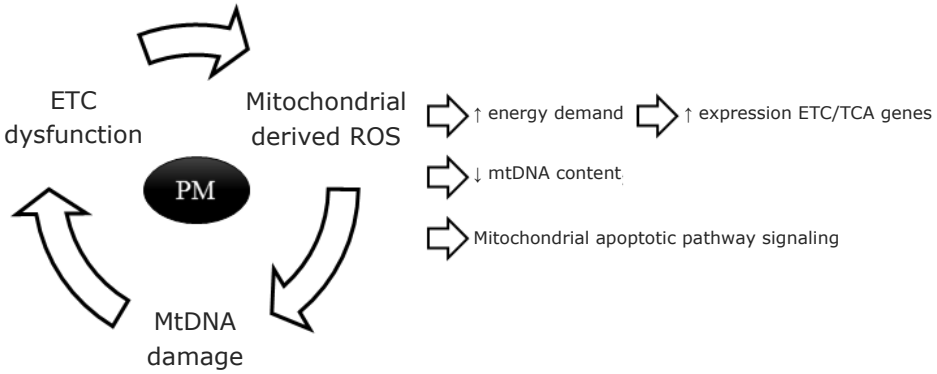

Figure 3. Schematic representation of the hypothesised pathway by which PM exposure alters mitochondrial functioning and genome maintenance. Within the mitochondria, PM can interact with the electron transport chain inducing increased levels of ROS production. ROS can damage mtDNA leading to further mitochondrial dysfunctioning and ROS production. Signalling of the presence of mitochondrial dysfunction to the nucleus may lead to an upregulation of electron transport genes to provide the required energy to repair or eliminate damaged cellular components. Elimination of damaged mtDNA and perturbation of mtDNA replication results in a reduction of mtDNA content. Eventually, accumulation of mitochondrial damage can lead to mitochondrial apoptotic signalling. TCA: tricarboxylic acid cycle; ETC: electron transport chain; mtDNA: mitochondrial DNA

Overall, differences were observed between the response to PM exposure in men and women. However, for both sexes interacting pathways are altered by $PM_{2.5}$ exposure; the mitochondrial apoptotic pathway is tightly regulated by several factors such as Bcl-2 family members, altered in women, and the PI3K/AKT (busulfan) pathway modulated in men.[221, 222] Moreover, the TCA cycle, deregulated in men, donates high-energy molecules to the ETC of which genes were differently expressed by PM exposure in women of both cohorts and in men of the validation cohort. In the validation cohort, the effects of air pollution on mtDNA content and expression levels of respiratory electron chain genes and genes contributing to the mitochondrial genome maintenance were more pronounced in women compared to men. Possibly, men are more effectively protected against environmental toxicants and ROS as implied by previous studies [223, 224]. Both studies reported more oxidative damage in female smokers compared to male smokers.[223, 224] In accordance, we observed in men exposed to relatively high PM levels, augmented expression levels of some aldehyde dehydrogenases (*ALDH7A1*↑, *ALDH1B1*↑), which convert reactive aldehydes (produced by oxidation of unsaturated fatty acids by ROS) to less toxic products, whilst in women *ALDH7A1* was down-regulated.

A strength of our study is that we validated genes in an independent validation cohort by means of qPCR. Moreover, in addition to gene expression, we analyzed mtDNA content in regard to PM exposure in the validation cohort. Our study has some limitations. First, observational studies do not allow to establish causality. Second, $PM_{2.5}$ estimates were only available for the validation cohort. Third, the large number of tests in combination with the observational study design reduces the power of the transcriptome-wide study. However, this was addressed through focused analyses on MitoCarta genes and mitochondrial pathways using the ORA approach.

Conclusions

Peripheral blood mtDNA content and expression of several genes related to mitochondrial genome maintenance, apoptosis and energy production were altered by PM exposure in a population of healthy middle-aged men and women, potentially reflecting mitochondrial and cellular damage. Future studies at

different omics level may further clarify the effect of air pollution on mitochondria functioning and biogenesis.

Authors' contributions

E Winckelmans contributed to the design of the study in close collaboration with TS Nawrot and K Vrijens. E Den Hond constructed the database. E Winckelmans performed the statistical analysis and, with contribution of M Tsamou, the bioinformatical analysis. TM de Kok and J Kleinjans were responsible for the transcriptome analysis. C Vanpoucke and W Lefebvre did the air pollution modelling. MtDNA content was measured by M Peusens. E Winckelmans wrote the first draft of the manuscript. All authors were involved in data interpretation and critical revision of the manuscript.

Funding

The project was funded by the Environment, Nature and Energy Department of the Flemish government (LNE/OL201100023/13034/M&G), Steunpunt Milieu- en Gezondheid and European Research Council (ERC-2012-StG 310898). Ellen Winckelmans has a PhD. fellowship of Hasselt University (BOF program). Karen Vrijens has a Postdoctoral Fellowship of the Research Foundation Flanders (12D7714N).

Supplementary material

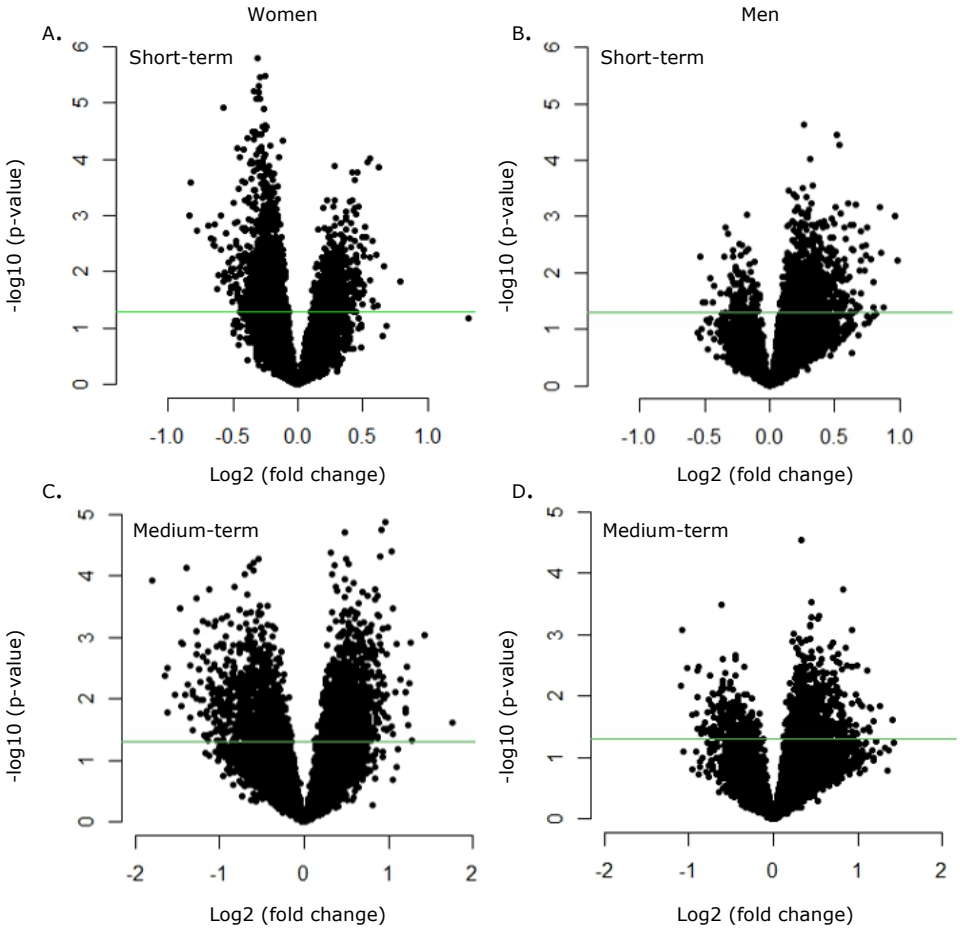

Figure S1. Volcano plots for short-term exposure (A: women, B: men) and for medium-term PM_{10} exposure (C: women, D: men). Log_2 fold changes are given for an increase in PM_{10} exposure of 10 $\mu g/m^3$.

Chapter 5

PERIPHERAL BLOOD TELOMERE LENGTH AND MITOCHONDRIAL DNA CONTENT IN RELATION TO OBESITY MEASURES: A POPULATION STUDY

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In preparation

Abstract

Background: Telomere length and mitochondrial DNA (mtDNA) content, both sensitive to oxidative stress and inflammation, are implicated in the onset and progression of several age-related diseases. However, the impact of obesity on telomere length and mtDNA content remains unclear. We assessed the sex-specific cross-sectional and longitudinal association of telomere length and mtDNA content with obesity indices in a general population.

Methods: In 678 randomly selected individuals from Flanders, Belgium (49.6% men, mean age 54.9 years), of whom 228 underwent a follow-up examination after an average 4.4 years, relative telomere length and mtDNA was measured in peripheral blood buffy coat.

Results: In a cross-sectional analysis an inverse linear trend was observed between telomere length and waist circumference (P=0.08). For a 1-SD increment in waist circumference over the follow-up time, telomeres were shortened by 3.16% (P=0.001) in both sexes, with stronger effect estimates in women (3.19%, P=0.01) compared to men (1.29%, P=0.44). We observed a positive trend between 4-year change in mtDNA content and waist circumference in men (P=0.05) and curvilinear association in women (P=0.01). For body mass index these associations did not reach statistical significance.

Conclusions: Our findings indicate that lower weight gain during aging is associated with a deceleration of telomere shortening and affect mtDNA content changes over time, especially in women.

Introduction

Obesity, defined by excessive fat accumulation, is a significant public health problem in industrialized countries. Excess adiposity is characterized by increased oxidative stress and inflammation and drastically increases a person's risk of developing aging-related diseases such as cardiovascular disease and diabetes.[225]

Several lines of evidence suggest that telomere attrition and mitochondrial function are sensitive to systemic inflammation and oxidative stress and are implicated in the key process of cellular aging.[45, 58, 226]

Telomeres are DNA tandem repeats of the sequence TTAGGG that cap the ends of mammalian chromosomes and protect chromosomal stability.[49] Every cell division telomeres become shorter until a critical stage is reached, eventually leading to replicative cell senescence or apoptosis.[50, 51] Besides telomere attrition by chronological aging, genetic background and cumulative exposure to inflammation and oxidative stress induced by environmental and lifestyle factors, including obesity, may accelerate telomere loss.[58-61] Telomere shortening may cause genomic instability and is associated with the aetiology of aging-related diseases including diabetes.[71, 74] It has been shown that maternal weight before pregnancy is associated with newborn telomere length.[227] Previous cross-sectional studies on the association between telomere length and obesity during adult life are controversial, some reported an inverse association[228-231] whilst others found no association[232-236]. Recently, three longitudinal observational studies found that telomere shortening during follow-up was associated with change in obesity measures over time, showing higher telomere attrition in persons gaining more weight.[237-239]

Mitochondria are the engines of cells and provide bioenergy in form of adenosine-5'-triphosphate via oxidative phosphorylation. Mitochondrial dysfunction may reduce energy supply and increase production of reactive oxygen species (ROS) which in turn may lead to cellular dysfunction and cell death. Each cell contains a different number of mitochondria from hundred to several thousand. A single mitochondrion harbours 2-8 copies of mitochondrial DNA (mtDNA) molecule.[40, 41] Alterations of mitochondrial DNA content are considered as a surrogate marker of mitochondrial functioning and damage.[45] Previous studies suggested that mitochondria are involved in the onset of age-related diseases.[46, 47, 240] Moreover,

obesity seems to coincidence with mitochondrial dysfunction and decreased mtDNA content.[236, 241-243] To our knowledge only one longitudinal study investigated the link between changes in adiposity and mtDNA content over time.[239]

Recent studies revealed a biological connection between telomere length and mitochondrial functioning.[51] Telomere dysfunction-induced p53 suppresses the expression of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and beta (PGC-1α and PGC-1) causing impaired mitochondrial functioning. Mitochondrial dysfunction in turn causes increased ROS production damaging both mitochondrial and telomere DNA.[51]

Epidemiological research studying both changes in telomere length and mitochondrial DNA over a follow-up period in association with changes in obesity measures is limited. In the current study, we assessed in both cross-sectional and longitudinal studies whether telomere length and mtDNA content measured in peripheral blood were sex-specifically associated with two obesity measures: waist circumference and body mass index (BMI). In the longitudinal design, we investigated whether changes in waist circumference and BMI over an average follow-up period of 4.4 years are accompanied by changes in telomere length and mtDNA content.

Methods

Study population

FLEMENGHO is an ongoing family-based population study that started in 1985 and has been described in detail previously.[244] Households (people with the same residential address) were randomly sampled in the northeastern part of Belgium by use of the SAS random function. The initial participation rate was 78.0%. Participants were repeatedly followed-up at a local examination center. At each examination, demographic data, life style factors and health parameters were provided through an extensive self-assessment questionnaire. BMI was calculated by dividing body weight in kilograms by squared height in meters. Obesity was defined as having a BMI greater than or equal to 30 kg/m^2. Waist circumference was measured in the horizontal plane midway between the lowest palpable rib and

the top of the iliac crest by study nurses. From 2005, both telomere length and mtDNA content were measured in peripheral blood of 678 participants. In 228 of these participants, telomere length and mtDNA content were measured at two examination phases with an average follow-up period of 4.4 years (range 2.8 to 5.7). This study was approved by the Ethics committee of the University of Leuven, and complies with the Helsinki declaration. All participants provided written informed consent at each contact.

DNA extraction

Genomic DNA was isolated from buffy coats of peripheral blood using the QIAmp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's guidelines. DNA yield and purity ratios (A260/280 and A260/230) of the isolated DNA were determined using a Nanodrop spectrophotometer (ND-1000; Isogen Life Science B.V., De Meern, the Netherlands).

Measurement of relative mtDNA content

Relative mtDNA content was measured, as described previously in detail,[245] by calculating the relative ratio of two mitochondrial sequences [mitochondrially encoded NADH dehydrogenase 1 (*MT-ND1)* and mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*)] to a single housekeeping nuclear gene (*RPLP0*)] using a qPCR assay. qPCR was performed using the 7900HT Fast Real-Time PCR System (Life Technologies, Foster City, CA, United States) in a 384-well format. Samples were run in triplicate and 6 inter-run calibrators were assessed on each reaction plate to account for inter-run variability. qBase software (Biogazelle, Zwijnaarde, Belgium) was used to normalize cycle threshold values of the two mtDNA sequences relative to the nuclear reference gene and to correct for run-to-run differences.[211] We achieved coefficient of variations between triplicate measurements within the same run of <0.5% for each of the amplified sequences, and 4.7% for the inter-run samples.

Measurement of relative telomere length

Relative leukocyte telomere length was measured, as described previously,[227] by means of a modified qPCR protocol. Briefly, we amplified the telomeric region,

using telomere specific primers (telg and telc), and one single-copy housekeeping gene (*RPLP0*) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, City, Country) in a 384-well plate. For consistency, samples were run in triplicate and inter-run calibrators were used to account for run-to-run differences. qBase software (Biogazelle, Zwijnaarde, Belgium) was used to normalize the cycle threshold values of the telomeric specific region relative to the cycle threshold values of the single-copy reference gene and to correct for run-to-run differences.[211] The coefficients of variation were 0.67% for telomere runs, 0.41% for single-copy gene runs and 8.8% for T/S ratios.

Statistical analysis

For database management and statistical analysis, we used SAS software, version 9.4. The two outcomes of interest (peripheral blood telomere length and mtDNA) were $\log_{10}$-transformed to obtain a normal distribution. For the cross-sectional analysis, continuous data were described as mean values ± SD or geometric mean (10^{th}-90^{th} percentile) and compared by a t test or a Wilcoxon rank sum test for normal distributed and non-normally distributed variables respectively. Categorical data are presented as frequencies (percent) and compared by a chi-square test. For the longitudinal analysis, distributions of continuous variables per examination were compared by a paired t test or Wilcoxon signed-rank test for normal distributed and non-normally distributed variables respectively. Categorical data were analysed by the McNemar's test.

In the cross-sectional analysis, we investigated the association of obesity indices with peripheral blood telomere length and mtDNA content. In the longitudinal analysis, we analysed the relation of within-subject change in telomere length and mtDNA content with change in waist circumference and BMI during the follow-up period. Within-subject changes were calculated by subtracting follow-up values by baseline values. For telomere length and mtDNA content, the difference between the $\log_{10}$-transformed values was considered as outcome, i.e. $\log_{10}$-transformed fold change (FC) in telomere length or mtDNA content where FC refers to the ratio of telomere length or mtDNA content at second examination and the value at first examination.

In both cross-sectional and longitudinal analyses, we used linear mixed models with family number as a random effect to account for non-independence of

observations within families. Models were adjusted for the following variables selected *a priori* based on previous literature and stepwise analysis in our cohort[35]: sex, age, current smoking status (non-smoker or current smoker), white blood cell (WBC) count, $\log_{10}$-transformed neutrophil to lymphocyte ratio (NLR). For mtDNA content, we additionally added platelet count and a quadratic term for age in the model as suggested by Knez et al. [245] In the longitudinal analysis, models fitting change in telomere length and mtDNA content were additionally adjusted for, respectively baseline mtDNA content or telomere length, changes in blood cell counts over time, and follow-up duration.

In our sensitivity analysis, we examined whether the observed associations were modified by sex by use of interaction terms and stratification. In addition, we categorized subjects as those with decreasing and increasing obesity measures over follow-up time and repeated longitudinal analyses after exclusion of participants with decreasing obesity measures.

We also explored the shape of the associations by using smoothing plots (natural cubic splines with number of knots based on Akaike Information Criterion).

For the cross-sectional analysis, results were back-transformed and presented as estimated percent difference in telomere length and mtDNA content for one standard deviation (SD) increase in the variable of interest. For the longitudinal analysis, results were presented as percent difference in fold change in telomere length or mtDNA content for one standard deviation (SD) increase in the variable of interest.

Results

Cross-sectional study

Characteristics of the study population

The study population consisted of 678 white Europeans of whom 336 (49.6%) were men (Table 1). Age ranged between 18 and 89 years. The proportion of smokers was similar for men (16.1%) and women (17.0%). Men and women had an average waist circumference of 98.9 cm (SD: 11.3 cm) and 91.5 cm (SD: 12.8 cm), respectively. 85 (25.3%) men were obese compared to 78 (22.8%) women.

Overall, telomere length was significantly longer (*P*=0.008) and mtDNA content significantly higher in women compared to men (*P*=0.0004).

Furthermore, we observed a significant association between telomere length and mtDNA content. Multivariable adjusted analysis showed that a 25% decrease (±IQR) in telomere length was associated with a 5.0% lower mtDNA content (*P*=0.003).

Table 1. Descriptive characteristics of the 678 participants in the cross-sectional analysis by sex

Characteristic	Men (n=336)[$]	Women (n=342)[$]	*P*-value*
Anthropometrics			
Age, years	54.56 ± 15.87	55.19 ± 14.82	0.60
Body mass index, kg/m^2	27.54 ± 3.95	26.83 ± 4.48	0.03
Waist circumference, cm	98.87 ± 11.27	91.46 ± 12.79	<0.0001
Systolic blood pressure, mmHg	132.96 ± 14.99	131.47 ± 18.41	0.25
Diastolic blood pressure, mmHg	83.65 ± 9.83	80.80 ± 9.47	0.0001
Questionnaire data			
Current smoking status	54 (16.1)	58 (17.0)	0.76
Current alcohol use	268 (79.8)	189 (55.3)	<0.0001
Anti-diabetic medication	21 (6.3)	19 (5.6)	0.70
Hypertensive	182 (54.2)	156 (45.6)	0.03
Obese	85 (25.3)	78 (22.8)	0.45
Substitution therapy		24 (7.0)	n.a.
Contraceptive pill intake		62 (18.1)	n.a.
Biochemical data			
Total cholesterol	4.86 (0.91)	5.26 (0.98)	<0.0001
LDL, mmol/l	2.94 ± 0.77	3.10 ± 0.88	0.02
HDL, mmol/l	1.28 ± 0.31	1.63 ± 0.37	<0.0001
Triglycerides, mmol/L	1.62 (0.88-3.06)	1.37 (0.81-2.40)	<0.0001
Blood glucose, mmol/L	4.98 (4.39-5.72)	4.82 (4.33-5.44)	0.0017
Serum creatinine (µmol/l)	96.13 (81.29-111.50)	78.70 (66.61-93.37)	<0.0001
Blood cell count			
White blood cells, x10^9/l	6.29 ± 1.46	6.54 ± 1.77	0.05
Neutrophil to lymphocyte ratio	2.00 (1.28-3.16)	1.76 (1.12-2.83)	<0.0001
Platelet, x10^9/l	214.96 ± 46.79	252.84 ± 59.58	<0.0001
Relative telomere length	0.95 (0.70-1.26)	0.99 (0.77-1.28)	0.008
Relative mtDNA content	0.94 (0.64-1.41)	1.03 (0.69-1.61)	0.0004

[$]Mean ± SD, geometric mean (10th-90th percentile) or number of subjects (%). *T test (continuous variables), Wilcoxon rank sum test (ordinal variable), chi-square test (categorical variables). MtDNA: mitochondrial DNA. N.a.: not applicable.

Obesity measures in association with peripheral blood telomere length and mtDNA content

We assessed whether peripheral blood telomere length and mtDNA content were associated with obesity measures by use of linear mixed models (Table 2).

Estimates are expressed as % difference per 1-SD increase in waist circumference (12.6 cm) and BMI (4.2 kg/m^2). Supplemental Figure S1 and S2 shows the corresponding scatterplots for waist circumference and BMI respectively. MtDNA content was not significantly associated with the assessed obesity measures (waist circumference: *P*=0.93, BMI: *P*=0.89). For a 1-SD increment in waist circumference, telomere length was 1.5% shorter (*P*=0.08). The effects did not significantly differ between men and women (*P* for interaction=0.29).

Table 2. Cross-sectional association of waist circumference and BMI with telomere length and mtDNA content

Outcome	Parameter	Sex	Estimate* (95% CI)	*P*	P_{int} sex
Telomere length					
	Waist circumference				
		Both	-1.46 (-3.05, 0.17)	0.08	0.29
		Men	-1.83 (-4.29, 0.69)	0.15	
		Women	-1.22 (-3.30, 0.91)	0.26	
	BMI				
		Both	-0.98 (-2.50, 0.56)	0.21	0.35
		Men	-1.45 (-3.78, 0.94)	0.23	
		Women	-0.78 (-2.75, 1.22)	0.44	
MtDNA content					
	Waist circumference				
		Both	0.12 (-2.42, 2.72)	0.93	0.76
		Men	1.21 (-2.74, 5.32)	0.55	
		Women	-0.25 (-3.62, 3.22)	0.88	
	BMI				
		Both	-0.17 (-2.56, 2.29)	0.89	0.61
		Men	0.91 (-2.83, 4.80)	0.64	
		Women	-0.56 (-3.70, 2.69)	0.73	

*Estimates expressed as % difference (95% CI) for a SD increase for waist circumference (SD: 12.6 cm) and BMI (SD: 4.2 kg/m^2). MtDNA : mitochondrial DNA. BMI: body mass index. CI: confidence interval. Analyses were adjusted for date of blood sampling, sex, age, current smoking status, WBC count, $\log_{10}$-transformed NLR, additionally for mtDNA content for platelet count and age^2. P_{int}: *P*-value interaction term.

Longitudinal study

Characteristics of the study population

Table 3 lists the characteristics by the examination phase of the 228 participants included in the longitudinal analyses. The mean follow-up duration was 4.4 years (range: 2.8-5.7). 119 (52.2%) participants were men. On average waist circumference, BMI, the number of obese participants as well as diastolic and systolic blood pressure increased significantly over follow-up period. Telomere length significantly decreased (*P*=0.0001) and mtDNA content tended to decrease

(*P*=0.12) over follow-up period. The distribution of telomere length and mtDNA content by examination phase is given in Figure 1A and 1B respectively. In 71.5% participants telomere length decreased over time. Change in telomere length was positively associated with mtDNA content changes: if FC of telomere length decreased by 15% (±IQR) then mtDNA content FC increased by 4.8% (*P*=0.04). Figure S3 shows the distribution of 4-year change in waist circumference and BMI in men and women.

Table 3. Descriptive characteristics of the 228 participants in the longitudinal analysis by examination phase

Characteristic	Examination 1[$]	Examination 2[$]	*P**
Anthropometrics			
Age, years	51.39 ± 15.05	55.79 ± 15.10	<0.0001
Body mass index, kg/m^2	26.36 ± 3.89	27.20 ± 4.03	<0.0001
Waist circumference, cm	91.13 ± 11.99	96.48 ± 12.07	<0.0001
Systolic blood pressure, mmHg	128.38 ± 15.46	134.13 ± 16.17	<0.0001
Diastolic blood pressure, mmHg	80.02 ± 9.86	83.48 ± 9.61	<0.0001
Questionnaire data			
Current smoking status	40 (17.54)	35 (15.35)	0.06
Current alcohol user	154 (67.54)	153 (67.11)	0.82
Anti-diabetic medication	9 (3.95)	16 (7.02)	0.008
Hypertensive	101 (44.30)	12 (56.58)	<0.0001
Obese	33 (14.47)	46 (20.18)	0.005
Substitution therapy [a]	7 (6.42)	11 (10.09)	0.16
Contraceptive pill intake [a]	29 (26.61)	24 (22.02)	0.13
Biochemical data			
Total cholesterol	5.21 ± 0.87	4.97 ± 0.94	<0.0001
LDL, mmol/l	3.16 ± 0.77	2.94 ± 0.81	<0.0001
HDL, mmol/l	1.44 ± 0.36	1.43 ± 0.40	0.77
Triglycerides, mmol/l	1.62 (0.89-3.09)	1.49 (0.80-2.57)	0.003
Blood glucose, mmol/l	4.88 (4.33- 5.55)	4.96 (4.39-5.77)	0.15
Serum creatinine, µmol/l	81.07 (64.53-100.78)	87.97 (71.80-108.19)	<0.0001
Blood cell count			
White blood cells	6.31 ± 1.60	6.28 ± 1.55	0.77
Neutrophil to lymphocyte ratio	1.90 (1.22-3.21)	1.85 (1.18-3.05)	0.23
Platelets, x10^9/l	231.75 ± 56.79	234.06 ± 56.11	0.37
Relative telomere length	1.05 (0.72-1.29)	0.97 (0.80-108.19)	0.0001
Relative mtDNA content	1.01 (0.70-1.49)	0.97 (0.66-1.47)	0.12

[$]Mean ± SD, geometric (10th-90th percentile) or number of subjects (%).*Paired t test (continuous variables), Wilcoxon signed rank test (ordinal variable), McNemar's test (categorical variables). [a]only women included (n=109). LDL: low-density lipoprotein. HDL: high-density lipoprotein. MtDNA: mitochondrial DNA.

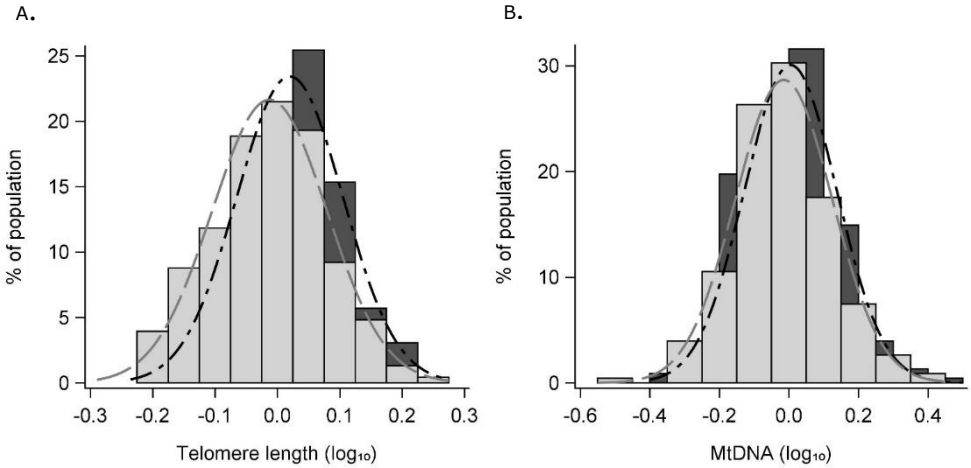

Figure 1. Distribution of (A) log_{10}-transformed telomere length and (B) mtDNA content in 228 participants at examination 1 (dark gray) and examination 2 (light gray). MtDNA: mitochondrial DNA content.

Changes in telomere length and mtDNA content in association with changes in obesity measures

Figure 2 shows scatterplots of changes in telomere length and mtDNA content by changes in waist circumference. Corresponding plots for BMI are given in supplemental figure S4. Table 4 lists the estimated % difference in FC of the outcome variable if change in waist circumference and BMI increases with 1-SD (Δ waist circumference: 7.1 cm, Δ BMI: 1.8 kg/m^2). While accounting for important covariates, a 1-SD increase in waist circumference and BMI over follow-up were linearly inversely associated with telomere length FC (-3.16%, *P*=0.001 and -2.00%, *P*=0.03, respectively). Although effect estimates were higher for women, no significant interaction between men and women was observed (*P* for interaction=0.65). FC in mtDNA content tended to be sex-specifically associated with change in waist circumference (see Figure 2 E-F). In men, we observed a positive association between FC in mtDNA content and waist circumference. In women, on the other hand, the association was nonlinear with a breakpoint around zero gain in waist circumference. For BMI, these associations did not reach a formal statistical significance. To overcome the nonlinear association of waist circumference and BMI with mtDNA content in women, we performed a sensitivity analysis in which we repeated the multivariable adjusted analyses excluding

persons with decreasing waist circumference and BMI over follow-up period (Table 4). For a 1-SD greater increase in waist circumference (7.1 cm) and BMI (1.8 kg/m^2) over follow-up, mtDNA content FC decreased by -7.8% (95% CI: -12.7, -2.7, *P*=0.005) and -5.0% (95% CI: -9.9, 0.2, *P*=0.06), respectively.

Correction for telomere length in the models analysing mtDNA content and vice versa did not alter the results substantially (results not shown).

Table 4: Association of Δ mtDNA content and Δ telomere length with Δ waist circumference and Δ BMI

		All participants			Sensitivity analysis§		
Parameter	Sex	Estimate*	*P*	P_{int} sex	Estimate*	*P*	P_{int} sex
Δ Telomere length							
Δ waist circumference							
	both	-3.16 (-4.99; -1.30)	0.001		-3.33 (-5.3; -1.31)	0.002	
	men	-1.29 (-4.54; 2.07)	0.44	0.65	-3.1 (-6.78; 0.71)	0.11	0.83
	women	-3.19 (-5.61; -0.72)	0.01		-3.18 (-5.82; -0.47)	0.02	
Δ BMI							
	both	-2.00 (-3.75; -0.22)	0.03		-1.9 (-3.83; 0.08)	0.06	
	men	0.51 (-2.16; 3.25)	0.71	0.10	0.39 (-2.79; 3.68)	0.81	0.18
	women	-3.31 (-5.69; -0.87)	0.009		-3.12 (-5.76; -0.4)	0.03	
Δ mtDNA content							
Δ waist circumference							
	both	-2.47 (-6.62; 1.87)	0.26		-5.79 (-10.41; -0.94)	0.02	
	men	8.37 (-0.01; 17.47)	0.05	0.05	5.97 (-4.26; 17.30)	0.25	0.06
	women	-5.77 (-10.59; -0.69)	0.03		-7.82 (-12.71; -2.65)	0.005	
Δ BMI							
	both	-1.57 (-5.50; 2.52)	0.44		-3.55 (-7.98; 1.10)	0.13	
	men	4.33 (-2.39; 11.51)	0.21	0.16	2.54 (-5.82; 11.64)	0.55	0.17
	women	-3.46 (-8.39; 1.74)	0.18		-5.00 (-9.91; 0.19)	0.06	

*Estimates expressed as % differences in fold change (95% CI) of telomere length and mtDNA content if the change in BMI and waist circumference increases with one std (Δ BMI: 1.8 kg/m2, Δ waist circumference: 7.1 cm). §Participants with decreasing BMI and waist circumference over follow-up duration are excluded (n=205). P_{int}: *P*-value interaction term.

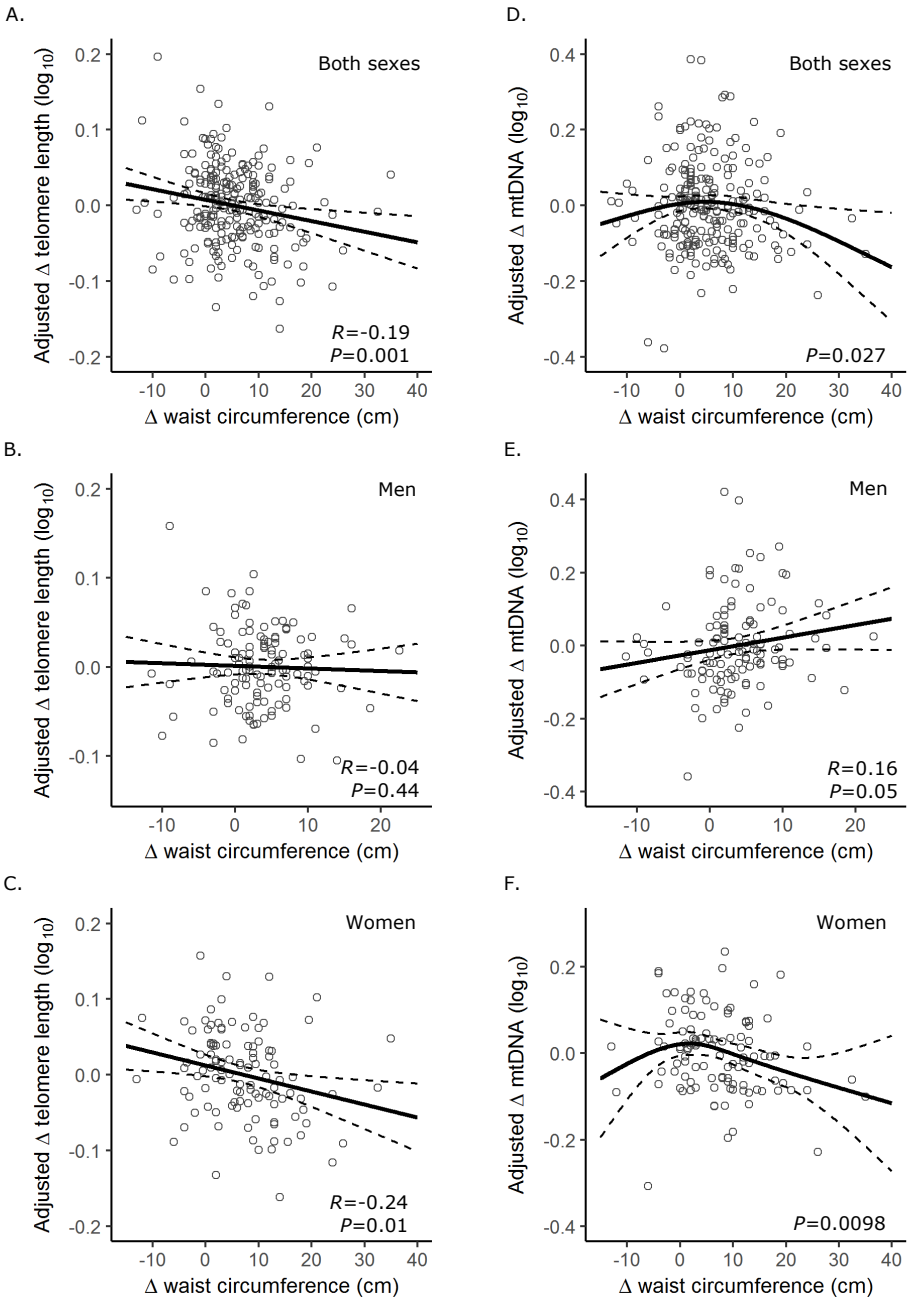

Figure 2. Within-subject change in waist circumference in association with change in telomere length (A-C) and mtDNA content (D-F). Adjusted for sex, age and a quadratic term for age (only for mtDNA), BMI, current smoking status, WBC count, platelet count (only for mtDNA content), $\log_{10}$ transformed NLR, and date of first blood sampling at first examination, if applicable, the changes of these variables over time and follow-up duration.

Discussion

In the current study, we investigated cross-sectional and longitudinal associations of telomere length and mtDNA content with obesity measures. Our study supports the idea that metabolic dysregulation (or excessive adiposity) is implicated in accelerated biological ageing. In a cross-sectional analysis, we found an inverse linear trend between waist circumference and telomere length but no evidence of association between waist circumference and mtDNA content. In the longitudinal study, we observed that a 4-year increase in waist circumference is accompanied by a higher telomere attrition rate, particularly in women. Change in mtDNA content was sex-specifically associated with change in waist circumference. In women, we found a curvilinear association between 4-year change in waist circumference and mtDNA content. In men, on the other hand, we observed a positive trend between change in waist circumference and mtDNA content over follow-up. Overall conclusions for BMI were similar but less strong compared to waist circumference. This is in line with earlier research in which they demonstrated that waist circumference is a better predictor for obesity-related health risks compared to BMI.[37, 246] By implementing waist circumference and BMI as continuous variables in the same regression model, Janssen et al.[37] demonstrated in a study population of 14,924 American adults that obesity-related health risk such as hypertension and hypercholesterolemia is explained by waist circumference, and not by BMI. Based on 1,000 Iranian adults, Hajian-Tilaki and Heidari[246] showed by means of receiver operating characteristic analysis that waist circumference exhibited a better discriminate performance than BMI for diabetes, particularly in women.

Obesity (excessive adiposity) is characterized by oxidative stress, which might be a consequence of obesity as well as a trigger of obesity. Obesity triggers oxidative stress by several mechanism such as increased tissue lipid levels, hyperglycemia, impaired antioxidant defences, chronic inflammation, and hyperleptinemia.[247] Increased ROS levels further induce the secretion of pro-inflammatory cytokines which in turn increase ROS, starting a negative feedback loop.[248] Moreover, obesity-induced oxidative stress may stimulate altered food intake and adipose tissue deposition worsening the condition and possibly leading to comorbidities of obesity.[248]

Since previous research implicated that telomere length and mtDNA content are associated with oxidative stress, inflammation and obesity-related diseases,[249, 250] it is important to evaluate associations of telomere length, mtDNA content with obesity measures in cross-sectional and longitudinal studies. There are several cross-sectional studies on the association between telomere length measured in peripheral blood and obesity measures. However, evidence is controversial: few studies did not observe any significant association,[232-236] whilst other reported an inverse association.[228, 237, 251, 252] For instance, a Swedish study of Nordfjäll et al [252] including 514 men and 475 women showed that telomere length is associated with waist circumference (P=0.03) and BMI (P=0.02) but only in women. Differences in protocols, study populations, anthropometric measures, statistical methods, inclusion of covariates in the analyses, and outcome of interest may affect study outcomes.

To our knowledge only three observational studies investigated whether telomere attrition rate was linked to change in obesity measures over follow-up time but not in a sex-specific manner.[237-239] Révész et al. observed in a study population of 1,808 adults of The Netherlands Study of Depression and Anxiety that greater 6-year increase in waist circumference was associated with larger peripheral blood telomere shortening (P=0.01).[237] Similar results were found in 70 young adults of the Bogalusa Heart study,[61] showing increased telomere attrition in participants with greater 11-year increase in BMI (P<0.001) and in 989 young adults from the Coronary Artery Risk Development in Young Adults Study[239] showing increased telomere shortening in association with greater 10-year waist circumference increase (P=0.04). Furthermore, two experimental studies reported significant increases in telomere length after a weight loss intervention.[253, 254] Changes in telomere length was inversely associated with changes in waist circumference and BMI during a 5-year nutritional intervention including 521 adults (55-80 years).[253] Garcia-Calzon et al. [254] reported that two months of an energy-restricted diet was associated with decreased telomere shortening (P<0.001) in a study population of 74 adolescents (12 -16 years).

Consistent with these previous studies, we observed a positive association between telomere shortening and 4-year increase in obesity measures. However, stratified analyses showed only a significant association in women.

Regarding mtDNA content, to date only two studies investigated the link between mtDNA content in peripheral blood and obesity in adults.[236, 241] Blood mtDNA content was inversely associated with BMI and waist circumference among 94 young Korean adults[241] and in a cohort study including 1700 female nurses.[236] To our knowledge only one study investigated whether a change in mtDNA content was associated with a change in obesity measures over a follow-up period.[239] In 989 participants from the Coronary Artery Risk Development in Young Adults Study[239] mtDNA was measured at the age of 15 and 25 years. They reported that greater waist circumference at 15 years of age predicted 10-year decrease in mtDNA content, however, no significant association was found between within-subject change in mtDNA content and waist circumference. In the current study, greater increase in waist circumference over time was associated with greater increase in mtDNA content in men. In women opposite effects were observed. Smoothing plots demonstrated a curvilinear relationship, with an inverse significant association between 4-year changes in waist circumference and mtDNA content in women who demonstrated increasing waist circumference over time. Adjustment for telomere length did not alter these findings. Whether increasing or decreasing mtDNA content is detrimental for human health is unclear. Both decreased and increased mtDNA content are associated with age-related diseases.[255-258] Moreover, whilst telomere length declines with age, a curvilinear association has been found between mtDNA content and age in the current study population[245] as well as in earlier research.[259]

The observed sex-specific associations in the current study may be due to differing fat storage patterns in men and women caused by metabolic an hormonal differences.[87] In the current study population, the range of 4-year change in waist circumference is broader in women compared to men which may also contribute to observed findings in men and women.

This study has several limitations that warrant consideration. Although we regressed the longitudinal change, observational studies do not allow to establish causality. Second, we cannot exclude the possibility of residual confounding by unknown factors associated with both obesity measures and telomere length or mtDNA content. Third, our study population consisted of only white Europeans so care must be taken when generalizing to other ethnicities.

Conclusion

In conclusion, our findings indicate that lower weight gain during aging is associated with decreased telomere shortening and affect mtDNA content changes over time, especially in women. We advise future longitudinal studies to check whether associations differ between subjects with increasing and decreasing obesity measures. Promoting normal weight by encouraging to eat healthy and to implement physical activity in daily life might help to prevent the onset and progression of age-related diseases.

Authors' contributions

E Winckelmans contributed to the design of the study in close collaboration with TS Nawrot and T Kuznetsova. J Knez constructed the database. E Winckelmans did DNA extractions, dilutions, quality control as well as mtDNA measurements using qPCR. E Winckelmans assisted D Martens in the telomere measurements. E winckelmans did the statistical analysis and wrote the first draft of the paper. All authors were involved in the revision of the manuscript.

Funding

The Fonds voor Wetenschappelijk Onderzoek Vlaanderen, Ministry of the Flemish Community, Brussels, Belgium, supported the Research Unit Hypertension and Cardiovascular Epidemiology (Leuven, Belgium) and Centre for Environmental Sciences, Hasselt University, (Diepenbeek, Belgium) (grants G.0734.09, G.0880.13 and G. 0881.13). The European Union also gave support to the Research Unit Hypertension and Cardiovascular Epidemiology (grants HEALTH-2011-278249-EU-MASCARA, HEALTH-F7-305507-HoMAGE, and ERC Advanced Grant-2011-294713-EPLORE) and to the Centre for Environmental Sciences (ERC-2012-stg 310898). EW has a BOF PhD.-fellowship (Bijzonder Onderzoeksfonds Hasselt University).

Supplementary material

Table S1. Change in telomere length in association with participants' characteristics

Parameter[b]	Estimate[a]	SE	*P*-value
Male sex	-2.35	1.84	0.19
Age, 15.1 years	-2.94	1.05	0.005
Current smoker	0.83	2.52	0.74
White blood cells, 1.60 x 10^9 cells/l	-1.88	1.09	0.082
Δ white blood cells, 1.48 x 10^9 cells/l	-1.18	1.11	0.29
Neutrophil to lymphocyte ratio (log_{10}), 0.16	2.27	1.04	0.031
Δ neutrophil to lymphocyte ratio (log_{10}), 0.15	1.69	1.08	0.12
Telomere length (log10), 0.09	-4.1	0.98	<.0001
Date blood sampling, 479.9 days	2.23	1.45	0.12
Follow-up duration, 216.3 days	-1.48	1.35	0.25

[a] Estimates expressed as % differences in fold change of telomere length.
[b] For continuous characteristics, the SD is given after the comma and estimates and corresponding SEs are expressed for a 1-SD increase in the explanatory variable. SE: standard error; SD: standard deviation.

Table S2. Change in mtDNA content in association with participants' characteristics

Parameter[a]	Estimate[b]	SE	*P*-value
Male sex	-6.2	4.37	0.14
Age, 15.1 years	27.65	12.62	0.042
Age^2, 15.1^2 years	-4.09	1.84	0.019
Current smoker	-4.87	5.77	0.37
White blood cells, 1.60 x 10^9 cells/l	-10.47	2.61	<.0001
Δ white blood cells, 1.48 x 10^9 cells/l	-9.75	2.54	0.0005
Platelets, 56.8 x 10^9 cells/l	6.41	2.44	0.011
Δ platelets, 38.9 x 10^9 cells/l	7.28	2.22	0.0016
Neutrophil to lymphocyte ratio (log_{10}), 0.16	-3.48	2.33	0.13
Δ neutrophil to lymphocyte ratio (log_{10}), 0.15	-4.65	2.44	0.051
MtDNA content (log_{10}), 0.13	-25.19	2.07	<.0001
Date blood sampling, 479.9 days	-4.32	3.14	0.16
Follow-up duration, 216.3 days	-2.94	3.08	0.34

[a] Estimates expressed as % differences in fold change of mtDNA content.
[b] For continuous characteristics, the SD is given after the comma and estimates and corresponding SEs are expressed for a 1-SD increase in the explanatory variable. MtDNA: mitochondrial DNA; SE: standard error; SD: standard deviation.

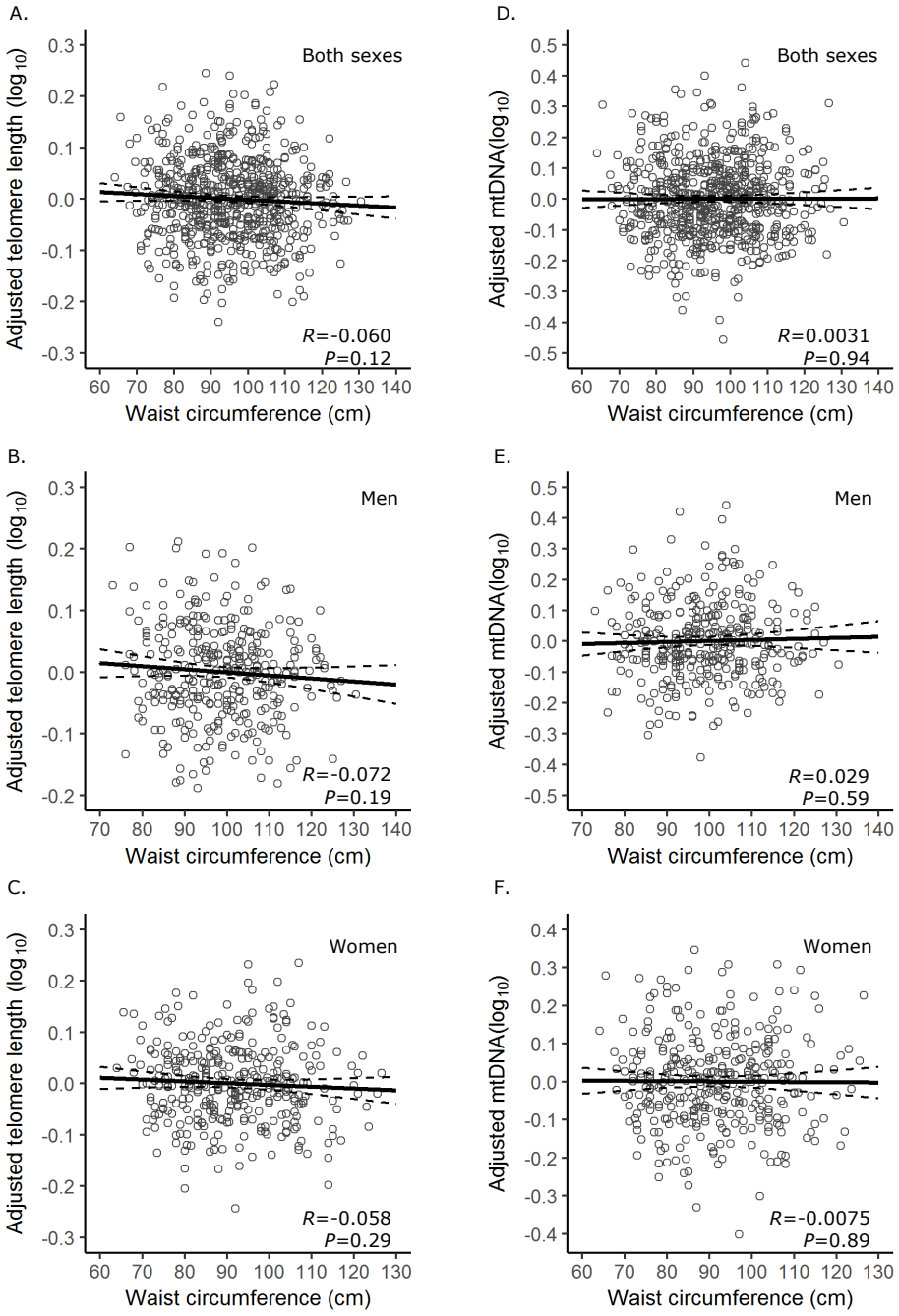

Figure S1. Waist circumference in association with telomere length (A-C) and mtDNA content (D-F). Adjusted for date of blood sampling, sex, age, current smoking status, WBC count, $\log_{10}$-transformed NLR, for mtDNA content additionally for platelet count and age^2.

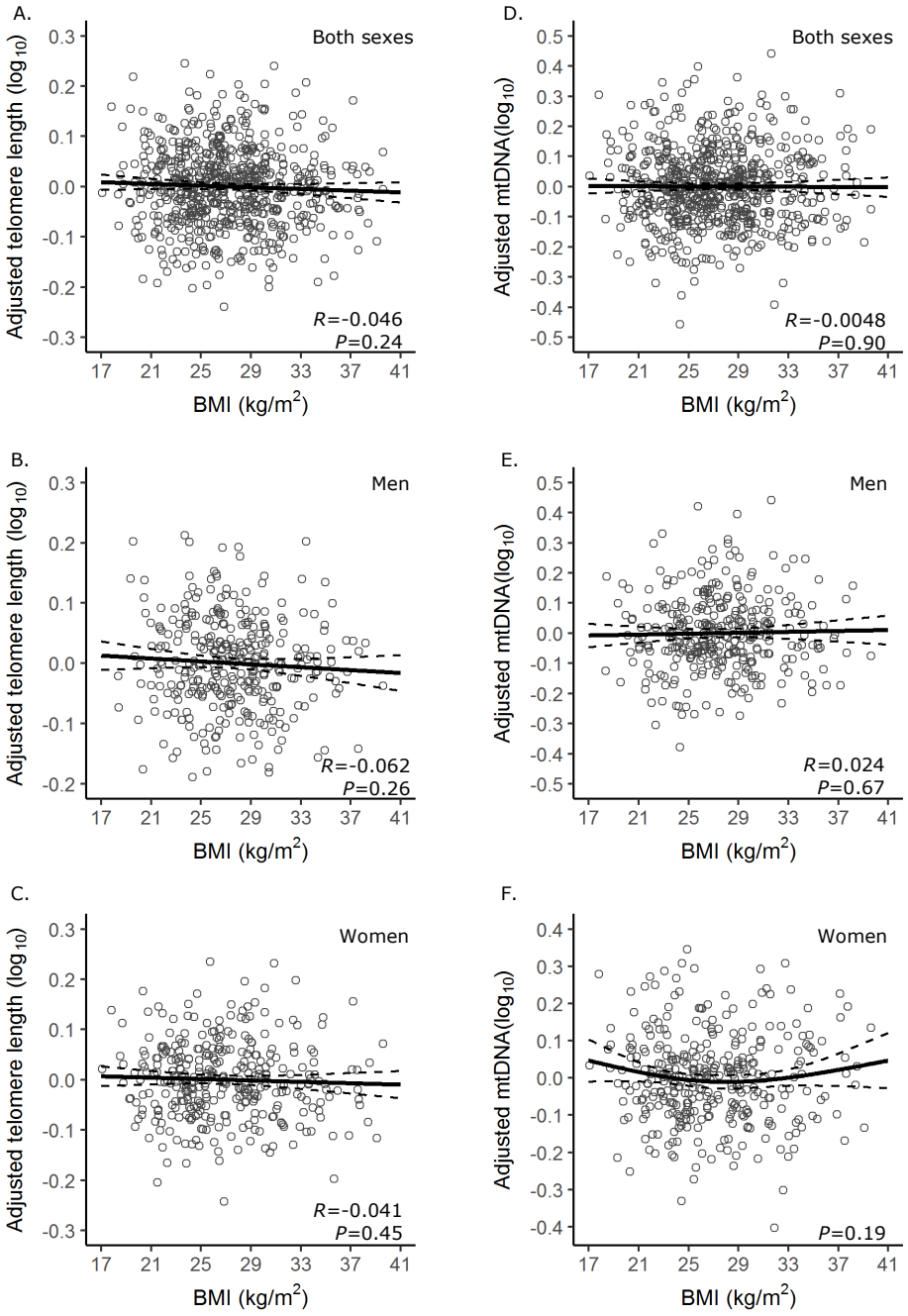

Figure S2. BMI in association with telomere length (A-C) and mtDNA (D-F). Adjusted for date of blood sampling, sex, age, current smoking status, WBC count, $\log_{10}$-transformed NLR, additionally for mtDNA for platelet count and age^2. BMI: Body mass index.

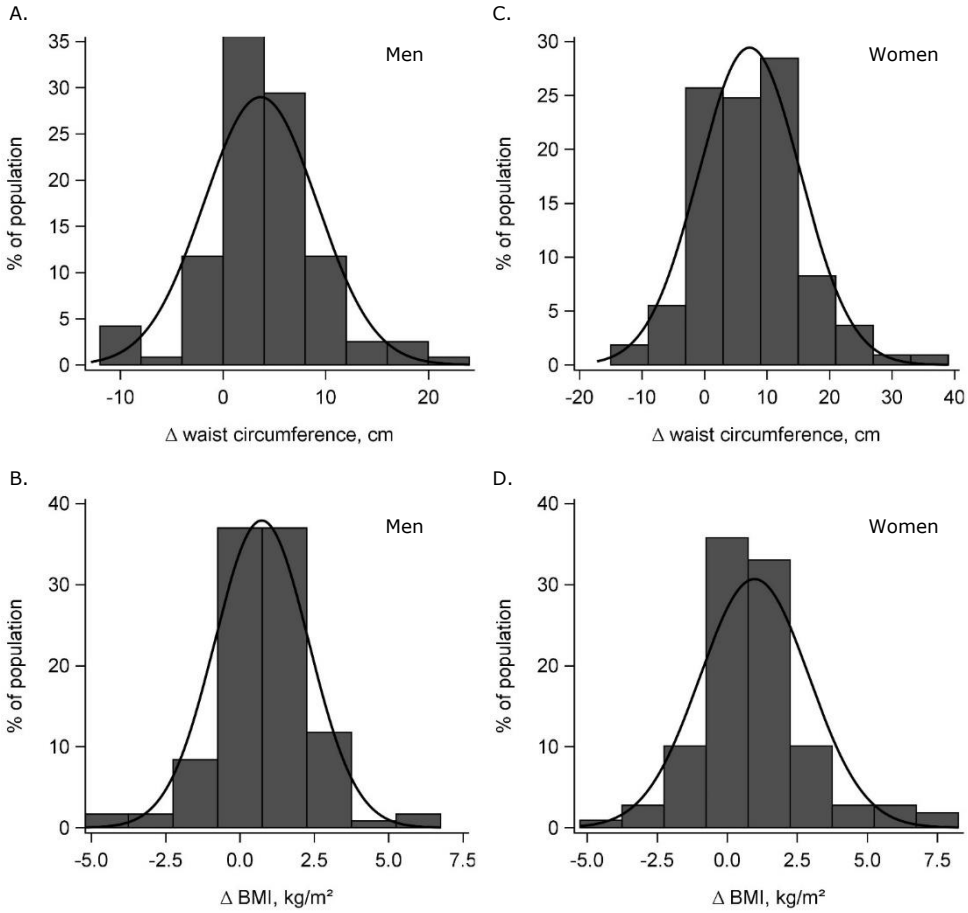

Figure S3. Distribution of within-subject change in waist circumference and BMI of (A, B) 119 men and (C, D) 109 women.

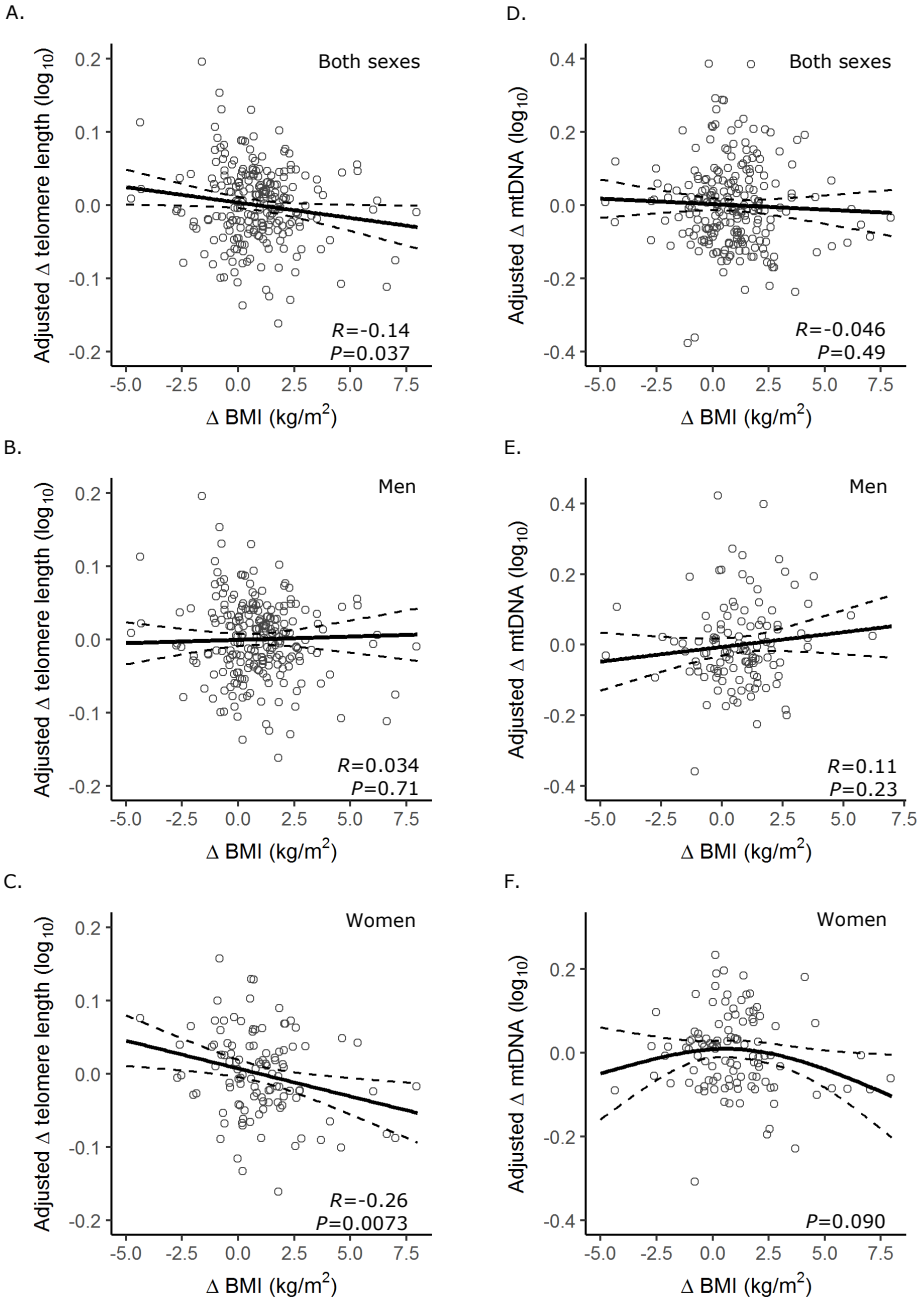

Figure S4. Within-subject change in BMI in association with change in telomere length (A-C) and mtDNA (D-F). Adjusted for sex, age [linear and quadratic term (only for mtDNA)], BMI, current smoking status, WBC count, platelet count (only for mtDNA), $\log_{10}$ transformed NLR, and date of first blood sampling at first examination, if applicable, the changes of these variables over time and follow-up duration.

Chapter 6

PERIPHERAL BLOOD MITOCHONDRIAL DNA CONTENT IN RELATION TO LONG-TERM PARTICULATE AIR POLLUTION EXPOSURE: A POPULATION STUDY

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In preparation

Abstract

Background: Mitochondrial DNA (mtDNA) is particularly susceptible to oxidative stress-induced damage, and therefore a potential important pathway for air pollution-linked diseases. We assessed the association between peripheral blood mtDNA content and long-term particulate matter <2.5 μm ($PM_{2.5}$) exposure in an adult population.

Methods: In 577 randomly selected individuals from Flanders, Belgium (50.6% men, mean age 55.5 years), of whom 222 were examined at two examination days with on average 4.4 years in between, relative mtDNA content was measured in peripheral blood buffy coat. We performed linear regression analyses adjusting for participants' characteristics to assess the association of long-term $PM_{2.5}$ exposure with mtDNA content and with the change in mtDNA content between the two measurements. Furthermore, we studied potential effect modification by sex, smoking status, and waist circumference.

Results: In the cross-sectional analysis, an interquartile range (IQR, 1.51 μg/m^3) increase in long-term residential $PM_{2.5}$ exposure was associated with a decrease in mtDNA content of 5.1% (P=0.03) with a more pronounced effect in abdominal obese participants (-6.6%, P=0.03) compared to non-obese participants (-3.2%, P=0.34). In a longitudinal analysis, a 1.51 μg/m^3 increment in residential $PM_{2.5}$ exposure was associated with a 10.9% (P=0.004) lower fold change of mtDNA over 4 years of follow-up. The association was stronger in smokers (-31.6%, P=0.004) compared to non-smokers (-8.9%, P=0.03) ($P_{interaction}$=0.04) and for participants with decreased (-0.08%, P=0.99) versus increased waist circumference over follow-up time (-13.6%, P=0.003) ($P_{interaction}$=0.04).

Discussion: We found an inverse association between long-term $PM_{2.5}$ exposure and peripheral blood mtDNA content in a general population. Our study suggests that future studies should take into account possible effect modification by (abdominal) obesity measures and potential synergism between smoking and environmental air pollution exposure.

Introduction

Fine particulate matter (aerodynamic diameter < 2.5 µm, $PM_{2.5}$) in ambient air has been linked with age-related pathogenesis including lung cancer, respiratory and cardiovascular diseases.[260] Production of reactive oxygen species (ROS) and oxidative stress are implicated to play a crucial role in the development of these prominent health effects.[261-264] ROS are mainly generated by the mitochondrial electron transport chain, which may be dysregulated by environmental exposures. Because of its proximal location, limited repair capacity and lack of histons and noncoding introns, mitochondrial DNA (mtDNA) is particularly susceptible to ROS-induced damage. Accumulation of mtDNA damage may trigger mtDNA degradation, mitochondrial fusion and fission, and mitochondrial destruction by mitophagy.[265] When the affected proportion of mitochondria exceeds a threshold this may lead to cellular dysfunction and in turn to cell death.[266]

Previous studies have indicated an altered mtDNA content in adults in response to air pollution exposure[216, 267-270] and smoking status[271, 272]. However, results were inconsistent with respect to the direction of the effects. Moreover, most studies focussed on working environments with high exposure levels or on specific subgroups such as diabetic patients[273]. Research of the impact of environmental particulate matter exposure on mtDNA content in a general population is still limited.

In the framework of the Flemish Study of Environment, Genes and Health Outcomes (FLEMENGHO), we assessed the association of long-term $PM_{2.5}$ exposure with mtDNA content and within-subject change of mtDNA content over a time period of ± 4 years. To understand better interindividual susceptibility, we studied potential effect modification by sex, smoking status, and waist circumference.

Methods

Study population

FLEMENGHO is an ongoing family-based population study that started in 1985 and has been described in detail previously.[244] Households (persons with the same residential address) were randomly sampled in the north-eastern part of Belgium

by use of SAS random function. The initial participation rate was 78.0%. Participants were repeatedly followed-up at a local examination center. At each examination, demographic data, life style factors and health parameters were provided through an extensive self-assessment questionnaire.

For 228 participants, mtDNA content was measured at two examination days with on average (range) 4.4 (2.8 to 5.7) years in between. During the second examination phase (2010-2013), we additionally measured mtDNA content in 340 participants resulting in 586 participants of which mtDNA content measurements were available for the second examination phase. This study was approved by the Ethics committee of the University of Leuven, and complies with the Helsinki declaration. All participants provided written informed consent at each contact.

DNA extraction and measurement of relative mtDNA content

Genomic DNA was isolated from peripheral blood buffy coats using the QIAmp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's guidelines. DNA yield and purity ratios (A260/280 and A260/230) of the isolated DNA were measured using a Nanodrop spectrophotometer (ND-1000; Isogen Life Science B.V., De Meern, the Netherlands). MtDNA content was measured, as previously described [245], by calculating the relative ratio of two mitochondrial sequences [mitochondrially encoded NADH dehydrogenase 1 (*MT-ND1)* and mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*)] to a single housekeeping nuclear gene (*RPLP0*) by use of a quantitative real-time polymerase chain reaction (qPCR) assay. The DNA samples were diluted to a concentration of 2.4 ng/µL in RNase free water. qPCR was performed by use of 2.5 µl extracted DNA and 7.5 µl master mix containing Fast SYBR Green dye 2x (Applied Biosystems, Inc., Foster City, California), forward and reverse primers, and RNase-free water. For consistency, samples were run in triplicate. Primers were diluted to a final concentration of 300 nM in the master mix. Each 384-well plate contained 2 no-template controls and 6 interrun calibrators. 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, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. qBase software (Biogazelle, Zwijnaarde, Belgium) was used to normalize

cycle threshold values of the two mtDNA sequences relative to the nuclear reference gene and to correct for run-to-run differences.[211]

Exposure measurement

$PM_{2.5}$ concentrations (μg/m³) for each participants' residential address were interpolated using a high resolution (25 × 25 m) spatiotemporal interpolation method. The interpolation model uses $PM_{2.5}$ data collected by a governmental stationary monitoring network (34 monitoring stations in Belgium) and takes into account land cover data obtained from satellite images (CORINE land cover data set). Coupled with a dispersion model (Immission Frequency Distribution Model)[97-99] that uses emissions from point sources and line sources, this model chain provides daily $PM_{2.5}$ values in a dense irregular receptor grid. Overall model performance was evaluated by leave-one-out cross-validation across monitoring stations. Validation statistics of the interpolation tool explained >80% of the spatiotemporal variability.[99] $PM_{2.5}$ exposure averaged over a five year period (2010-2014) was considered representative for participants' long-term $PM_{2.5}$ exposure. Participants' residential addresses are marked in Figure 1, showing the north-eastern part of Belgium.

Other measurements

On the day of the examinations, trained nurses measured the participants' blood pressure and anthropometric characteristics. Waist circumference was measured with a tape measure midway between the lowest palpable rib and the top of the iliac crest. High waist circumference (as a measure of abdominal obesity) was defined as a waist circumference equal or higher than 102 cm for men and higher than 88 cm for women.[39] Body mass index (BMI) was calculated by dividing body weight in kilograms by squared height in meters. Obesity was defined as having a BMI greater than or equal to 30 kg/m^2. Blood pressure was calculated as the average of five consecutive auscultatory readings measured with a standard mercury sphygmomanometer according to European guidelines.[276] Participants with a measured blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or use of antihypertensive drugs were categorized as being hypertensive. At the examination day, venous blood samples were drawn after participants had been fasting for at least 6 hours. Plasma glucose, serum levels of creatinine,

triglycerides, and total and high-density lipoprotein cholesterol were measured by automated methods in a single certified laboratory. Diabetes mellitus was determined by self-reported diagnosis, fasting glucose level exceeding 7.0 mmol/L (126 mg/dl), or use of antidiabetic agents.

Statistical analysis

MtDNA content was $\log_{10}$-transformed to obtain a normal distribution.

In the cross-sectional analysis, we investigated the association between long-term $PM_{2.5}$ exposure and mtDNA content of the second examination phase. Categorical data are described as frequencies (percent) and compared by a chi-square test. Continuous data are given as mean values ± SD or geometric mean (10^{th} to 90^{th} percentile) and compared by a t test or a Wilcoxon rank sum test for normally and non-normally distributed variables respectively.

In the longitudinal analysis, we analysed the relation between long-term $PM_{2.5}$ exposure and within-subject change in mtDNA content during the follow-up period. Within-subject changes were calculated by subtracting follow-up values by baseline values. For mtDNA content, the difference between the log-transformed values was considered as outcome, i.e. log-transformed fold change in mtDNA content where fold change refers to the ratio of mtDNA content at second examination and the value at first examination. Data are described per examination phase: categorical variables were compared by a McNemar's test and distributions of continuous variables were compared by a paired t test or a Wilcoxon signed-rank test for non-normally distributed variables.

In both analyses, we used linear mixed models with residential address number as a random effect to account for non-independence of observations living in the same household. Models were adjusted for the following *a priori* selected variables: sex, age (linear and quadratic term), current smoking status (non-smoker or current smoker), white blood cell (WBC) count, $\log_{10}$-transformed neutrophil to lymphocyte ratio (NLR) and platelet count. In the longitudinal analysis, models were additionally adjusted for baseline mtDNA content, changes in blood cell counts over time, and follow-up duration.

In secondary analyses, we examined whether the observed associations were modified by sex, current smoking status (non-smoker vs smoker), and waist

circumference (normal vs high) by use of interaction terms and stratification. In the longitudinal analysis, we additionally assessed effect modification by within-subject change in waist circumference over follow-up duration (decreased or increased). We also explored the shape of the associations by using smoothing plots (natural cubic splines with number of knots based on Akaike Information Criterion).

For the cross-sectional analysis, results were back-transformed and presented as estimated percent difference in mtDNA content for one interquartile range (IQR) increase in long-term $PM_{2.5}$ exposure. For the longitudinal analysis, results were presented as difference in fold change in mtDNA content.

Because nine participants with relatively high exposure levels (>15 μg/m^3) may influence the results, especially in subgroup analyses, we excluded these participants resulting in a final study population of 577 participants for the cross-sectional study and 222 participants for the longitudinal study. In a sensitivity analysis, we checked whether inclusion of the nine relatively highly exposed participants in the main model changed the obtained results.

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC) and RStudio (R version 3.3.2).

Results

Cross-sectional study

Characteristics of the study population

The 577 white European participants included 292 (50.6%) men, 137 (23.7%) obese participants and 87 (15.1%) current smokers (Table 1). The average (range) was 55.5 (20-89) years. Waist circumference differed significantly between men (average: 99.4 cm, SD: 10.6 cm) and women (average: 92.4 cm, SD: 12.6 cm). 119 (40.8%) men and 176 (61.8%) women had a high waist circumference (≥102cm for men and ≥88cm for women). In total 284 (49.2%) participants were hypertensive including 44.1% of the male participants and 57.2% of the female participants. MtDNA content was significantly higher in women compared to men (P=0.0002). Mean (IQR) long-term $PM_{2.5}$ was 12.4 (1.51) μg/m^3. For all participants, exposure levels were below the European

annual mean limit value (25 μg/m^3) but above the limit of the World Health Organization (WHO) air quality guidelines (10 μg/m^3).

Table 1. Descriptive characteristics of the 577 participants in the cross-sectional analysis by sex

Characteristic	Men (n=292)[$]	Women (n=285)[$]	*P*-value*
Anthropometrics			
Age, years	55.49 ± 14.86	55.42 ± 14.12	0.95
Body mass index, kg/m^2	27.63 ± 3.73	26.89 ± 4.46	0.03
Waist circumference, cm	99.38 ± 10.60	92.37 ± 12.60	<0.0001
Systolic blood pressure, mmHg	132.52 ± 14.36	131.46 ± 17.55	0.43
Diastolic blood pressure, mmHg	83.83 ± 9.65	81.44 ± 9.53	0.0029
Questionnaire data			
Current smoking status	47 (16.1)	40 (14.40)	0.49
Current alcohol use	234 (80.1)	163 (57.2)	<0.0001
Diabetic	20 (6.8)	14 (4.9)	0.32
Hypertensive	130 (44.5)	154 (54)	0.01
Obese	73 (25.0)	64 (22.5)	0.43
High waist circumference [a]	119 (40.8)	176 (61.8)	<0.0001
Substitution therapy		19 (6.7)	n.a.
Contraceptive pill intake		48 (16.8)	n.a.
Biochemical data			
Total cholesterol	4.83 ± 0.89	5.26 ± 0.98	<0.0001
LDL, mmol/l	2.93 ± 0.75	3.10 ± 0.90	0.020
HDL, mmol/l	1.28 ± 0.31	1.66 ± 0.37	<0.0001
Triglycerides, mmol/L	1.61 (0.88-2.90)	1.33 (0.80-2.27)	<0.0001
Blood glucose, mmol/L	4.98 (4.39-5.72)	4.83 (4.33-5.44)	0.0065
Serum creatinine (μmol/l)	97.37 (82.15-111.50)	79.62 (68.34-93.37)	<0.0001
Blood cell count			
White blood cells, x10^9/l	6.29 ± 1.42	6.45 ± 1.69	0.20
Neutrophil to lymphocyte ratio	2.00 (1.28-3.13)	1.73 (1.12-2.78)	<0.0001
Platelet, x10^9/l	214.12 ± 46.85	253.56 ± 58.96	<0.0001
Relative mtDNA content	0.94 (0.64-1.41)	1.04 (0.70-1.61)	0.0002

[$]Mean ± SD, geometric mean (10th-90th percentile) or number of subjects (%). *T test (continuous variables), Wilcoxon rank sum test (ordinal variable), chi-square test (categorical variables). [a] Waist circumference higher than 102 cm for men and 88 cm for women. LDL: low-density lipoprotein; HDL: high-density lipoprotein; mtDNA: mitochondrial DNA; n.a.: not applicable.

Association between mtDNA content and long-term $PM_{2.5}$ exposure

We investigated whether peripheral blood mtDNA content is associated with long-term $PM_{2.5}$ exposure in 577 participants and whether there is effect modification by sex, current smoking status, and waist circumference (Table 2). Estimates are expressed as % difference per 1-IQR (1.51 μg/m^3) increase in $PM_{2.5}$ exposure. We observed a significant association between long-term $PM_{2.5}$ exposure and mtDNA

content (-5.1%, 95%CI: -9.35, -0.66%, *P*=0.03). Interaction terms of long-term $PM_{2.5}$ exposure with sex, smoking status and waist circumference were not significant. Nevertheless, we see stronger associations in participants with high waist circumference.

Table 2. Cross-sectional analysis between mtDNA content and long-term $PM_{2.5}$ exposure

Subgroup	N	Estimates*	*P*-value	*P-value*$_{int}$
Total	577	-5.10 (-9.35, -0.66)	0.03	
Men	292	-4.18 (-10.91, 3.05)	0.21	0.78
Women	285	-5.51 (-11.40, 0.77)	0.08	
Non-smokers	490	-4.95 (-9.67, 0.02)	0.05	0.58
Smokers	87	-6.51 (-16, 4.05)	0.21	
Normal waist circumference	282	-3.18 (-9.55, 3.65)	0.34	0.66
High waist circumference	295	-6.64 (-12.35, -0.56)	0.03	

*Estimates expressed as % difference (95% CI) in mtDNA content for an IQR increase in $PM_{2.5}$ (IQR: 1.51 μg/m^3).
P-value$_{int}$: *P*-value interaction term.

Figure S1A shows the smoothing plot for the association between long-term $PM_{2.5}$ exposure and mtDNA content. The association was flat at lower $PM_{2.5}$ levels, and steepest above 13 μg/m^3.

Longitudinal study

Characteristics of the study population

Table 3 lists the characteristics of the 222 participants by the examination phase. Follow-up duration was on average 4.4 years with a range between 2.8 to 5.7 years. 117 (52.7%) participants were men. Age at first examination had a mean (range) of 51.9 (18.8 to 82.1) years. On average waist circumference increased with 5.4 cm during follow-up period (*P*<0.0001). $PM_{2.5}$ exposure had a median (IQR) of 12.6 (1.6) μg/m^3. The Spearman correlation between mtDNA content on both examination days was low (*R*=0.13, *P*=0.05).

Table 3. Descriptive characteristics of the 222 participants in the longitudinal analysis by examination phase

Characteristic	Examination 1[$]	Examination 2[$]	*P*-value*
Anthropometrics			
Age, years	51.88 ± 14.88	56.27 ± 14.94	<0.0001
Body mass index, kg/m^2	26.46 ± 3.88	27.31 ± 3.99	<0.0001
Waist circumference, cm	91.45 ± 11.87	96.83 ± 11.86	<0.0001
Systolic blood pressure, mmHg	128.43 ± 15.45	134.21 ± 16.24	<0.0001
Diastolic blood pressure, mmHg	79.92 ± 9.75	83.42 ± 9.60	<0.0001
Questionnaire data			
Current smoking status	38 (17.12)	34 (15.32)	0.10
Current alcohol user	150 (67.57)	148 (66.67)	0.64
Diabetic	11 (4.95)	17 (7.66)	0.014
Hypertensive	98 (44.14)	127 (57.21)	<0.0001
Obese	33 (14.86)	46 (20.72)	0.0046
High waist circumference [a]	74 (33.3)	117 (52.7)	<0.0001
Substitution therapy [b]	7 (3.15)	10 (4.50)	0.26
Contraceptive pill intake [b]	26 (11.71)	22 (9.91)	0.21
Biochemical data			
Total cholesterol	5.22 (0.88)	4.98 (0.94)	<0.0001
LDL, mmol/l	3.17 (0.77)	2.95 (0.81)	<0.0001
HDL, mmol/l	1.43 (0.35)	1.43 (0.40)	0.85
Triglycerides, mmol/l	1.63 (0.91-3.09)	1.50 (0.80-2.57)	0.004
Blood glucose, mmol/l	4.90 (4.39-5.55)	4.97 (4.39-5.77)	0.17
Serum creatinine, µmol/l	80.94 (64.53-100.78)	88.08 (71.79-108.19)	<0.0001
Blood cell count			
White blood cells	6.30 ± 1.61	6.27 ± 1.56	0.78
Neutrophil to lymphocyte ratio	1.90 (1.22-3.21)	1.85 (1.18-3.05)	0.27
Platelets, x10^9/l	231.59 ± 56.84	233.76 ± 55.89	0.41
Relative mtDNA content	1.02 (0.70-1.49)	0.96 (0.66-1.45)	0.07

[$]Mean (± SD), geometric mean (10%-90% interval) or number of subjects (%).*Paired t test (continuous variables), Wilcoxon signed rank test (ordinal variable), McNemar's test (categorical variables). [a] Waist circumference higher than 102 cm for men and 88 cm for women. [b]only women included (n=105); LDL: low-density lipoprotein; HDL: high-density lipoprotein; mtDNA: mitochondrial DNA.

Association between change in mtDNA content and long-term PM2.5 exposure

We investigated whether within-subject change in mtDNA content was associated with long-term exposure to $PM_{2.5}$. Participants exposed to 1.51 µg/m^3 higher $PM_{2.5}$ exposure are estimated to have a 10.8% (95%CI: 3.9, 17.3%, *P*=0.004) lower FC in mtDNA content over the follow-up period (Table 4). Although the association was not found to be sex-specific ($P_{interaction}$=0.68), sex-stratification showed a significant association only in women (-14.1%, 95%CI -22.8, -4.4%, *P*=0.01).

Furthermore, the association tended to be different for smokers compared to non-smokers ($P_{interaction}$=0.04) and for participants with decreased versus increased waist circumference over follow-up time ($P_{interaction}$=0.04). Among smokers and non-smokers respectively, an increase in $PM_{2.5}$ of 1.51 µg/m^3 is associated with a decrease in mtDNA FC of 31.6% (95%CI:12.5, 46.6%, *P*=0.004) and 8.9% (95%CI: 0.8, 16.5%, *P*=0.03). The association between $PM_{2.5}$ exposure and mtDNA FC was only significant in participants with increased waist circumference during follow-up (-13.6, 95%CI: -20.4, -6.2%, *P*=0.003) but not in participants with decreasing waist circumference (-0.08, 95%CI: -22.4, 28.71, *P*=0.99).

Table 4. Analysis between change in mtDNA content and long-term $PM_{2.5}$ exposure

Subgroup	N	Estimates*	*P*-value	*P-value*$_{int}$
Total	222	-10.83 (-17.26, -3.90)	0.004	
Men	117	-7.69 (-16.68, 2.28)	0.12	0.68
Women	105	-14.09 (-22.78, -4.42)	0.01	
Non-smokers	188	-8.94 (-16.45, -0.76)	0.03	0.04
Smokers	34	-31.66 (-46.61, -12.49)	0.004$	
Waist circumference decreased over follow-up	34	-0.08 (-22.43, 28.71)	0.99	0.04
Waist circumference increased over follow-up	188	-13.59 (-20.44, -6.16)	0.003$	
Normal waist circumference	105	-9.25 (-19.68, 2.53)	0.12	0.81
High waist circumference	117	-13.09 (-20.76, -4.67)	0.003$	

*% difference (95% CI) in mtDNA FC for an increase of 1-IQR of long-term $PM_{2.5}$ (IQR: 1.51 µg/m^3). *P-value*$_{int}$: *P*-value interaction term. $FDR-adjusted *P*-values < 0.05. °$PM_{2.5}$ < 15 µg/m^3.

Figure S1B shows the smoothing plot for the association between long-term $PM_{2.5}$ exposure and within-subject change of mtDNA. The observed negative association is mostly driven by exposure above 13 µg/m^3.

In a sensitivity analysis, we repeated the main analyses with inclusion of the participants with $PM_{2.5}$ estimates > 15 µg/m^3, which did not alter the reported associations in our main analysis substantially. Figure 1 shows the scatterplots of the cross-sectional and longitudinal association with and without exclusion of these participants.

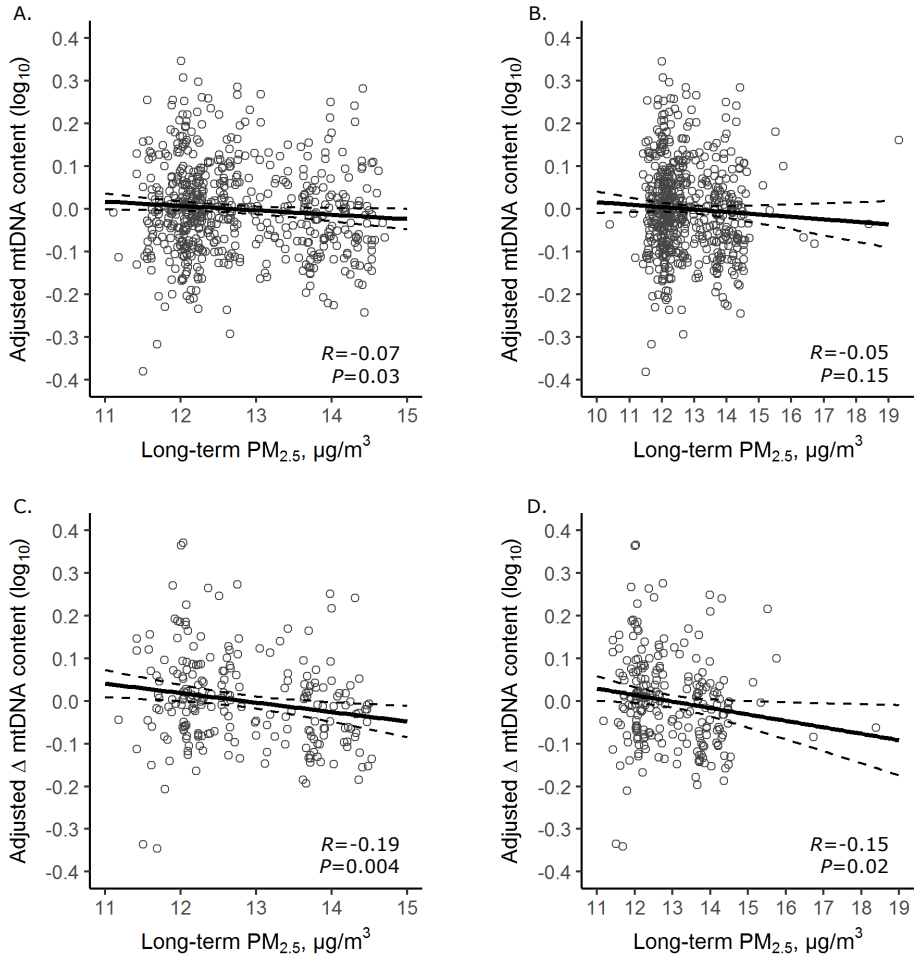

Figure 1. Scatterplot of mtDNA content (A,B) and within-subject changes in mtDNA content (C,D) by long-term $PM_{2.5}$ exposure, excluding (A, C) and including (B, D) participants with $PM_{2.5}$ estimates > 15 µg/m^3. Multivariable linear analyses were adjusted for sex, age (linear and quadratic term), current smoking status, WBC count, platelet count, log_{10} transformed NLR and, for the model fitting change in mtDNA content, additionally for baseline mtDNA content, change in blood cell counts, and follow-up duration. Residential address was included as random intercept. Dotted lines represent 95% CI. R: correlation coefficient.

Discussion

Our key finding is that peripheral blood mtDNA content and within-subject change in mtDNA content over a 4-year follow-up period is inversely associated with long-term residential $PM_{2.5}$ exposure in a general population sample. In the cross-sectional study, stratified analyses showed stronger effect sizes in participants with high waist circumference. The longitudinal study showed a more pronounced association between change in mtDNA content and long-term $PM_{2.5}$ exposure in women, smokers, and participants with high waist circumference (abdominal obesity). Furthermore, excluding participants with decreasing waist circumference over follow-up period resulted in greater effect sizes. Loss in waist circumference may have several underlying reasons, such as disease and diet and sport program, which themselves may alter mtDNA content over time.

The underlying biological mechanisms by which air pollution exposure contributes to adverse health effects are not well understood, although oxidative stress is hypothesized to play a central role. Increased ROS production due to air pollution exposure may result in a depletion of mtDNA content. The observed study findings are consistent with previous studies on ambient air pollution exposure. The Beijing Truck Driver Air Pollution Study[267] showed that mtDNA content was inversely associated with personal elemental carbon exposure during work hours and with 5-day and 8-day ambient PM_{10} exposure. Previously, we observed a negative association between peripheral blood mtDNA content and 1-week, 1-month and 2-year PM_{10} and $PM_{2.5}$ averages in 150 adults of the third Flemish Environment and Health Survey.[269] A recent study on 646 elderly male participants in the Normative Aging Study (Greater Boston)[270] demonstrated a negative association between mtDNA abundance and 1-year averaged $PM_{2.5}$ mass. On the other hand, a Swedish study[268] found a positive association between mtDNA content and personal respiratory dust exposures above 0.7 mg/m^3 measured during working hours among welders, and Hou and colleagues[216] observed a positive association between mtDNA content and averaged PM (PM_{10}, PM_1, PM_{10}-PM_1) exposure during work hours in 63 male steel workers in Italy. Inconsistency between study findings may be due to variations in composition and size of particles, exposure concentration levels, and exposure duration. Most previous studies[216, 267, 268] studied short-term particulate matter exposure, which may not capture

cumulative effects caused by prolonged environmental air pollution exposure. In the current study, we observed more pronounced associations for specific population subgroups, suggesting that differences in population characteristics between studies may further contribute to inconsistencies between study findings. In line with previous findings,[277-281] abdominal obese participants seemed to be more vulnerable to air pollution exposure. The observed synergistic interaction may be explained by the fact that both air pollution as well as obesity are associated with increased inflammation and oxidative stress. Moreover, obesity is shown to enhance the effects of air pollution on inflammatory markers.[281, 282]

Furthermore, in the longitudinal study, smokers showed higher effect sizes. However, due to the limited number of smokers (n=34), this finding needs to be interpreted with caution. Additional studies on populations with more smokers are necessary to support potential synergism between smoking and particulate matter exposure.

We recognize some limitations of our study. First, as with all observational studies, we cannot establish causality and we cannot exclude the possibility of residual confounding by some unknown factor that is associated with both peripheral blood mtDNA content and long-term $PM_{2.5}$ exposure estimates. However, extensive self-assessment questionnaires in combination with collection of biochemical and clinical measures by trained staff provided the necessary information to correct for several person-related characteristics.

Second, the low correlation of blood mtDNA between both time points suggests high biological variability over time. Our findings were in line with a previous study on 63 male healthy workers in Italy in which a correlation coefficient of 0.13 was reported between peripheral blood mtDNA content of the first day and the fourth day of the work week.[216]

At last, our study focussed on the North-eastern part of Belgium and, since exposure levels and composition of particles is region dependent, study findings may not be generalizable to other regions of Europe. Moreover, our study population included only white Europeans so care must be taken when generalizing to other ethnicities.

Conclusion

Overall, we found a negative association between long-term $PM_{2.5}$ exposure and peripheral blood mtDNA content in a general population. Our study indicates that future studies should take into account potential effect modification by (abdominal) obesity measures and potential synergism between smoking and environmental air pollution exposure.

Authors' contributions

E Winckelmans contributed to the design of the study in close collaboration with TS Nawrot and T Kuznetsova. J Knez constructed the database. E Winckelmans did DNA extractions, dilutions, quality control as well as mtDNA measurements using qPCR. E winckelmans did the statistical analysis and wrote the first draft of the paper. All authors were involved in the revision of the manuscript.

Funding

The Fonds voor Wetenschappelijk Onderzoek Vlaanderen, Ministry of the Flemish Community, Brussels, Belgium, supported the Research Unit Hypertension and Cardiovascular Epidemiology (Leuven, Belgium) and Centre for Environmental Sciences, Hasselt University, (Diepenbeek, Belgium) (grants G.0734.09, G.0880.13 and G. 0881.13). The European Union also gave support to the Research Unit Hypertension and Cardiovascular Epidemiology (grants HEALTH-2011-278249-EU-MASCARA, HEALTH-F7-305507-HoMAGE, and ERC Advanced Grant-2011-294713-EPLORE) and to the Centre for Environmental Sciences (ERC-2012-stg 310898). EW has a BOF PhD.-fellowship (Bijzonder Onderzoeksfonds Hasselt University). Dr Cox is a postdoctoral fellow of the Flemish Scientific Fund supported by grant 12Q0517N.

Supplementary material

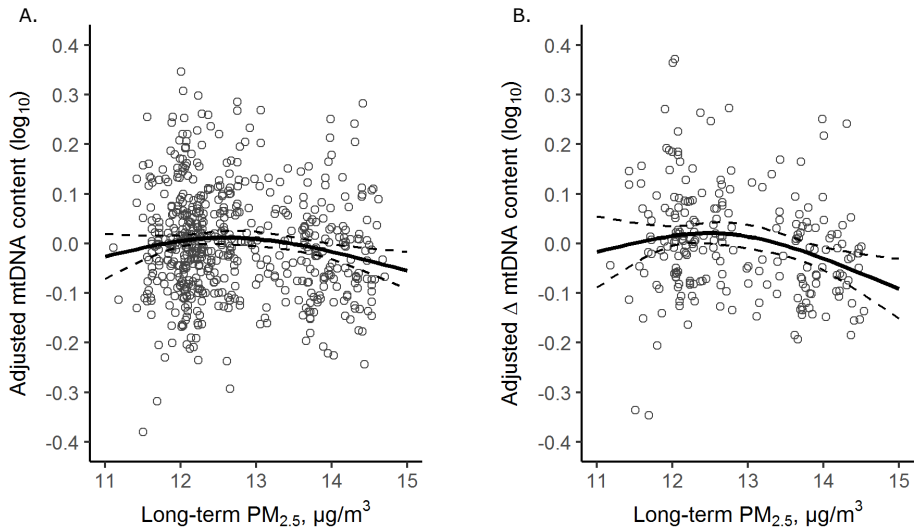

Figure S1. Scatterplots and cubic smoothing splines of mtDNA (A) content and within-subject changes in mtDNA content (B) by long-term $PM_{2.5}$ exposure. Analyses were adjusted for sex, age and a quadratic term for age, BMI, current smoking status, WBC count, platelet count, log_{10} transformed NLR and, for the model fitting change in mtDNA content, additionally for follow-up duration. Residential address was included as random intercept. Dotted lines represent 95% CI.

Chapter 7

GENERAL DISCUSSION

Chronic age-related diseases are the main cause of disability and death worldwide. However, the question why some people die prematurely and others survive healthy to old age remains largely unanswered. From early life onwards, we are continuously exposed to a plethora of environmental inputs and lifestyle factors which affect, along with genetic factors, the aging process. During the last few decades, the impact of excess body fat (as a marker of sedentary lifestyle and high-calorie food intake) and air pollution exposure on human health have become of growing concern in our modern society. In this context, we attempted to elucidate potential biological pathways that may intermediate the effect of environmental exposures on the development of chronic diseases. In a life-course concept, we studied environmental stressors during different stages of life including prenatal development and middle and late adulthood.

The novelties of this dissertation are:

1. The exploration of the shape of the association between gestational PM_{10} exposure and fetal growth and of potential effect modification by gestational age
2. Assessing the impact of gestational $PM_{2.5}$ exposure on the fetal transcriptome
3. Studying the impact of PM on the adult mitochondria-related transcriptome and validation of findings in an independent study population
4. Assessing the impact of $PM_{2.5}$ exposure on changes of peripheral blood mtDNA content over a follow-up period of ±4 years
5. Evaluation of the effect of obesity indices on both mtDNA content and telomere length in a longitudinal study design

Impact of particulate air pollution

PM air pollution is an omnipresent environmental risk factor for public health worldwide. Since the mechanisms driving air pollution-induced adverse health effects are poorly understood, in this doctoral dissertation, we tried to further elucidate underlying biochemical pathways by means of hypothesis-driven and hypothesis-generating approaches.

Fetal growth

Fetal growth is an important indicator of adverse health effects later in life. Mapping environmental exposures that influence fetal growth is therefore of great concern. Maternal air pollution exposure during pregnancy may affect the fetus in two different ways: 1) indirectly, through mediation by inflammatory effects on the mother's cardiorespiratory system and 2) directly, after translocation of (ultra)fine particles via the mother's bloodstream to the placenta or into the amniotic fluid. Wick *et al.*[149] showed in an *ex vivo* human placental perfusion model that polystyrene particles with a diameter up to 240 nm are able to cross the placental barrier. Previous studies investigated the impact of ambient air pollution on a range of pregnancy outcomes such as small for gestational age, birth weight, low birth weight, premature birth, and stillbirths.[22, 24, 25] Although there is growing evidence for a negative association between air pollution and fetal growth, several reviews[22, 105] and meta-analyses[106, 108] noted substantial heterogeneity between published studies regarding the study population, effect size, methodology, air pollutant of interest, and outcome of interest. A review of Stieb *et al.*[108] suggested to further explore the variation in effects by exposure window. Most studies only considered longer exposure windows such as entire pregnancy and trimesters, not taking into account the distribution of air pollution during pregnancy. For example, consistently moderate and low air pollution levels with occasional high air pollution exposure levels may both result in similar average exposure levels.[283] Therefore, besides assessing entire pregnancy and trimester exposures, we analysed in **chapter 2** shorter time windows during critical stages of development and fetal growth, including the perinatal period,[127] early embryogenesis,[284, 285] and last month of pregnancy.[105] Whereas most previous studies only considered term births, we also included extremely and moderately preterm births in order to assess potential effect modification by

gestational duration. Another novel aspects of this study is the exploration of the shape of the association between fetal growth measures and PM exposure.

In our study of nearly 530,000 newborns, we observed significant associations of gestational PM_{10} exposure with birth weight and being small for gestational age (SGA) among neonates born after 31 weeks of gestation. For both of these outcomes, associations were considerably stronger in babies born moderately preterm than for those born at term. For a 10 $\mu g/m^3$ higher maternal PM_{10} exposure averaged over the entire pregnancy, birth weight was estimated to be 24.0 g (95% CI: 20.9, 27.2 g) and 39.0 g (95% CI: 26.4, 51.5 g) lower among term and moderately preterm babies respectively. For SGA, corresponding odds ratios were 1.09 (95% CI: 1.06, 1.12) and 1.19 (95% CI: 1.07, 1.32) respectively.

In addition, segmented regression models showed that the assessed associations deviated from linearity with a breakpoint around 35 $\mu g/m^3$. Stronger effects of PM_{10} exposure on fetal growth were observed at lower concentration with a flattening out of the slope at higher exposure concentrations. As suggested by Ambrose and Barua,[133] the nonlinear shape of the observed associations may be caused by saturation of the underlying biochemical and cellular processes at higher exposure levels.

Mitochondria and air pollution exposure

Both in the hypothesis-generating and hypothesis-driven approaches applied in this doctoral dissertation, the importance of mitochondria in the response to ambient air pollution exposure during different stages of life is emphasised.

In a subset of 142 newborns of the ENVIR*ON*AGE birth cohort, we elucidated sex-specific pathways linked to maternal $PM_{2.5}$ exposure during pregnancy **(Chapter 3)**. Maternal $PM_{2.5}$ exposure during the last month of pregnancy was associated with an upregulation of the electron transport chain (ETC) and tricarboxylic acid (TCA) cycle pathway, however, only in boys. Previously, Janssen *et al.*[92] observed, in an independent subset of 174 newborns of ENVIR*ON*AGE, a negative association between placental mtDNA content and PM_{10} exposure during the last month of pregnancy. Both studies highlight the role of mitochondria as biosensors of PM exposure during prenatal life. In **chapter 4** where we analysed transcriptome-wide data of 98 adults, pathway analyses revealed a positive association of

medium-term (one month averages before blood sampling) PM_{10} exposure with the expression of genes encoding proteins of the ETC in women and of the TCA cycle in men. Short-term (one week averages before blood sampling) PM_{10} exposure was linked to pathways implicated in mitochondrial genome maintenance and apoptosis in women. We validated several ETC genes and genes involved in the mitochondrial genome maintenance by means of qPCR in an independent study population of 169 adults providing more confidence in the robustness of our obtained results. Furthermore, we observed a negative association between peripheral blood mtDNA content and PM exposure for both men and women in the validation study. These results were consistent with the findings of **Chapter 6**, showing a negative association between mtDNA content and long-term $PM_{2.5}$ exposure in adults. In addition, this study demonstrated a negative association between long-term $PM_{2.5}$ exposure and change in mtDNA content over a follow-up period of around 4 years. Previous studies assessing the association between mtDNA content and particulate air pollution are listed in Table 1. Studies are heterogeneous regarding the population of interest, exposure window (short- versus long-term) and measurement of PM exposure (outdoor PM vs PM at workplace vs personal monitoring). For short-term PM exposure, both positive and negative associations have been observed. However, positive associations were only found for PM exposure measured at workplace where PM exposure is relatively high.[216, 268] Moreover, Xu *et al.*[268] demonstrated a non-linear association in welders: below a respirable dust exposure (particulate diameter < 4 µm) of 0.7 mg/m^3 no significant association was found (β: -0.031, 95% CI: -0.47, 0.41, *P*-value: 0.89, n=57) whereas above 0.7 mg/m^3 a borderline positive association was reported (β: 0.037, 95% CI: -0.00075, 0.075, *P*-value: 0.054, n=41). For long-term outdoor exposure all studies reported decreased mtDNA content with increasing PM exposure.[269, 270, 286]

Regarding gene expression, human studies on mitochondrial response to particulate air pollution are lacking. Nevertheless, male mice exposed to cigarette smoke for 8 weeks showed an upregulation of a range of mitochondria-linked genes including genes encoding a component of the ATP synthase and succinate dehydrogenase complex.[287] Using western blots and enzyme assays, they showed that cigarette smoke exposure elicited an upregulation of the expression of complexes II, III, IV, and V and of the activity of complexes II, IV, and V of the

ETC. In accordance with these study results, Hoffmann and colleagues observed significantly increased ETC protein levels (Complex II, III, V) in the human bronchial epithelial cell line BEAS-2B in response to cigarette smoke exposure for 6 months.[214]

Altogether, our study findings along with previous study results support the following hypothesis that PM air pollution alters ETC functioning causing increased ROS production. In turn, elevated ROS levels can damage mtDNA, leading to further mitochondrial dysfunctioning and ROS production. To repair or eliminate damaged cellular components, the electron transport genes are upregulated to provide the required energy. Elimination of damaged mtDNA and perturbation of mtDNA replication results in a reduction of mtDNA content. Eventually, accumulation of mitochondrial damage can lead to mitochondrial apoptotic signalling.

The parallel findings of this doctoral dissertation among newborns and adults regarding both transcriptomic pathways as well as mtDNA content indicate that mitochondria are important targets of environmental risk factors in a life-course context, as mitochondria were altered by PM exposure during different stages of life.

Table 1. Summary of epidemiological studies on particulate air pollution exposure and mtDNA content

Author, year	Area	N	Study characteristics	Exposure window	PM monitoring and PM average	Direction of effect
Adults – peripheral blood						
Hou *et al.*, 2010[216]	Brescia, Italy	63	- Steel workers - 100% men - 27-55 years old	Work hours	- Work place - PM_{10}: ~233 μg/m^3	Positive
Hou *et al.*, 2013[267]	Beijing, China	240	- Beijing Truck Driver Air Pollution Study - 120 truck drivers and 120 office workers - 66.7% men - 31.9 ± 6.8 years old	8-day	- Outdoor in Beijing - PM_{10}: ~120 μg/m^3	Negative
Pieters *et al.*, 2016[286]	Genk, Belgium	166	- 46.4% men - 60-80 years old	1-year	- Outdoor at residential address - $PM_{2.5}$: ~21.1 μg/m^3	Negative
Xu *et al.*, 2017[268]	Southern Sweden	101	- welders - 100% men - 23-60 years old	Work hours	- Work place - RD: ~1.1 mg/m^3	Positive[b]
Winckelmans *et al.*, 2017[269]	Flemish region, Belgium	150	- Third Flemish Environment and Health Survey - 44.4% men - 50-65 years old	1-week, 1-month, 1-year[a]	- Outdoor at residential address - PM_{10}: 23.7 μg/m^3 - $PM_{2.5}$: 15.8 μg/m^3	Negative
Peng *et al.*, 2017[270]	Greater Boston, the USA	646	- Normative Aging Study from Greater Boston - 100% men - 51-100 years old	1-year	- Outdoor at residential address - $PM_{2.5}$: ~10.24 μg/m^3	Negative
Wong *et al.*, 2017[288]	Xuanwei and Fuyuan, China	148	- Xuanwei Exposure Assessment Study - 100% women - 20-80 years old	1-day	- Personnel monitoring - $PM_{2.5}$: ~ 167 μg/m^3	Negative
Winckelmans *et al.* (in preparation)	Flemish region, Belgium	577	- FLEMENGHO - 50.6% men - 20-89 years old	5-year	- Outdoor at residential address - $PM_{2.5}$: 12.4 μg/m^3	Negative
Newborns - placenta						
Janssen *et al.*, 2012[92]	North-eastern Belgium	174	- ENVIR*ON*AGE - 46.1% boys	Last week, last month and third trimester[a] of pregnancy	- Outdoor at residential address - PM_{10}: 24.4 μg/m^3	Negative

[a]Exposure window for which mean or median exposure is given. [b]Borderline non-significant (P=0.054) for welders exposed to high levels of respirable dust (>0.7 mg/m^3, n=41) and not significant considering all participants (P=0.33). RD: respirable dust.

Transcriptomic signatures and air pollution exposure

The characterisation of transcriptomic profiles in response to PM air pollution may identify novel biomarkers of exposure as well as biomarkers prognostic for exposure-related adverse health effects emerging later in life. In addition, it may provide a leap forward in unravelling the molecular mechanisms driving PM-induced adverse health effects.

In **chapter 3**, we assessed sex-specific transcriptomic responses to gestational $PM_{2.5}$ exposure in a subset of 142 newborns of the ENVIR*ON*AGE birth cohort for which whole genome gene expression was measured using Agilent microarrays. Apart from mitochondrial changes discussed in the previous section, several PM-responsive pathways were identified that have previously been linked with exposure-related adverse health outcomes including neurological and cardiovascular disorders, and cancer. Both for girls and boys, expression of olfactory receptors was downregulated by $PM_{2.5}$ exposure during the last month of pregnancy. Previously, a review[289] including 18 human studies summarized evidence indicating that uptake of air pollutants via the nose may directly damage olfactory epithelium (including the olfactory receptors) by inducing DNA damage and cause cytotoxic and inflammatory changes in the olfactory bulb and cortex which may cause olfactory dysfunction. In our study investigating PM exposure during pregnancy, fetuses are exposed to air pollution via the mother. Besides indirect PM effects on the fetus or uptake of pollutants in the fetal circulation, ultrafine pollutants may penetrate into the amniotic fluid.[290] From thereon air pollution may pass through the developing nose, can bind olfactory receptors, and initiate signal transduction down the olfactory sensory neurons to the olfactory bulb. Hence, signals can be further transmitted to higher regions of the brain. Along this same pathway, air pollutants can be retrogradely transported and cause damage to the brain.[170, 289] Since olfactory dysfunction has previously been linked with decreased quality of life and may precede major neurodegenerative complications[291, 292] and death in elderly,[293, 294] the observed PM-induced olfactory receptor gene expression alterations in newborns may indicate a role of the olfactory signaling pathway intermediating the association between early life PM exposure and development of neurological issues later in life. Although our study only provides suggestive evidence, it may motivate future observational and

experimental studies to further assess PM-induced changes of the olfactory signal transduction pathway in fetuses and newborns. Regarding the nervous system, we additionally observed altered expression of neurotransmitter receptor encoding genes and genes essential for axon guidance development in boys in response to respectively short- and long-term (annual average before delivery) $PM_{2.5}$ exposure. We identified interconnected DNA damage pathways (related to the *TP53* gene network) for boys and girls that may underlie the carcinogenic potential of $PM_{2.5}$ exposure in early life. It is plausible that deregulated gene expression of key players of the DNA damage response may increase disease susceptibility later in life. Furthermore, in girls the expression of defensins was generally lower with increasing long-term $PM_{2.5}$ exposure. Defensins are major components of the innate immunity and a decreased level of these antimicrobial peptides have been associated with higher susceptibility to infection in preterm babies.[188, 189]

In **chapter 4**, we explored the association of short-term (one week) and medium-term (one month) PM_{10} exposure on the transcriptome of 98 middle-aged men and women living in Flanders, Belgium. Since previous studies demonstrated the importance of mtDNA as target of environmental exposures and as potential intermediate biomarker of exposure-chronic disease associations, the goal of this study was to further elucidate PM exposure-linked expression changes of genes encoding proteins that are (partly) localized in the mitochondria (i.e. the Human MitoCarta geneset). So instead of an hypothesis-generating approach analysing gene expression of the whole genome as in **chapter 3**, we have chosen for a more targeted approach focussing on a subset of 1064 Human MitoCarta genes. As discussed in the previous section, our study findings indicate that the main functions of mitochondria, including energy production and regulation of apoptosis, were altered by PM exposure.

Although we observed potential PM responsive genes/pathways that were previously linked with environmental exposure-related diseases, further studies are necessary to: 1) validate our study results, 2) investigate whether expression changes of the identified genes in blood are representative for changes in target organs, 3) investigate whether expression of these genes can be used as an intermediate marker of health effects, 4) assess transcriptome effect sizes that are of importance in terms of identifying people at risk for chronic diseases, 5)

investigate whether changes in gene expression are cumulative in terms of exposure, and 6) see whether 'transcriptome memory' exists for the identified genes (i.e. if alterations in gene expression remain during different life stages by e.g. epigenetic alterations).

In conclusion, the transcriptomic studies of this doctoral dissertation aid in the elucidation of molecular mechanisms underlying the association between PM exposure and adverse health effects and provided new hypotheses that may inspire future epidemiological and experimental research in the field of environmental health risk assessment.

Multiple hypothesis testing

The emergence of high-throughput omics technologies has led to the so-called p >> n paradigm.[295] Under this new paradigm, the number of variables p (i.e. number of genes in transcriptomics) that is analysed is much larger than the number of independent subjects n. Typically thousands of hypotheses are tested and the number of subjects is often limited due to technical and economical limitations of the study. Analysis of many hypotheses in separate univariate models, each prone to a decision error, requires strong adjustments for multiple testing such as the implementation of Bonferroni correction,[296] false discovery rate correction, [297] and more recently a Bayesian approach of correction[298]. In the epidemiological context of this doctoral dissertation, the variability among the transcriptome is likely to depend on the exposome (of which we were specifically interested in PM exposure), endogenous factors, and the individual's genetic background. Since most of the influencing factors are not known and not controlled for, the unexplained variability in the dataset along with the relatively small range of the exposure of interest compared to experimental studies, makes it challenging to identify associations between PM exposure and gene expression after correction for multiple testing. To this end, we applied pathway analyses to identify gene sets, rather than individual genes, associated with PM exposure. We did overrepresentation analyses without multiple testing correction to observe subtle continuous effects (as expected for air pollution exposure in an epidemiological study). In addition gene set enrichment analyses was performed which uses the fold change to identify significant pathways rather than *P*-values thereby overcoming the issue of multiple testing. Furthermore, pathway analyses

as well as a thorough literature study of the identified genes and pathways may provide a biological plausible interpretation of the transcriptomic dataset.[77] The correction of multiple testing is less strict if one defines a priori a gene set of interest as in **chapter 4** where we focused on the Human MitoCarta gene set. Although omics and multiple testing correction are inseparable according to many experts, we argue that, in the area of molecular epidemiology, validation in independent study cohorts strengthens the reliability of the obtained study results. As previously described, in **chapter 4,** we assessed a subset of 13 differentially expressed genes in an independent validation study population by means of qPCR. However, study findings of **chapter 3** still need be confirmed in a validation study.

Impact of excess body fat on telomere length and mitochondrial DNA content

Increasing obesity rates, especially for central obesity, poses a major public health challenge since they increase the risk for non-communicable diseases (including diabetes mellitus, cardiovascular and fatty liver diseases, and cancer) and premature mortality.[4] Furthermore, it has considerable financial implications for health systems.[4] Obesity is characterized by the presence of excessive adipose tissue which is identified to increase systematic inflammation and oxidative stress, both interacting with telomere attrition and mitochondrial functioning and structure.[299] In addition, the oversupply of cells with excess glucose and lipids has been shown to fragment mitochondria, increase mitochondrial reactive oxygen species production, and promote mtDNA damage accumulation.[300] Previously, our research group investigated the impact of maternal pre-pregnancy body mass index (BMI) on newborn's telomere length.[227] For each 1-kg/m^2 increase in maternal pre-pregnancy BMI the estimated decrease in cord blood and placental telomere length was 0.50% (95% CI: 0.17, 0.83%, *P*=0.003) and 0.66% (95% CI, 0.25, 1.06%, *P*=0.002) respectively. In a next step, we wanted to investigate the association of obesity measures with mtDNA content and telomere length in an adult population (**chapter 5**). Furthermore, we analyzed whether within-subject changes in mtDNA content and telomere length over a follow up-period of around 4 years parallels changes in obesity measures. Besides assessing BMI, the

most frequently used indicator of obesity, we considered waist circumference as a measure for central obesity. In the cross-sectional analysis, a 1-SD higher waist circumference was associated with a 1.46% (95% CI: 0.17, 3.05%, *P*=0.08) higher telomere shortening. Similar results have been found by several previous studies on adults.[228, 237, 251, 252] Recently, three observational[237-239] and two experimental studies[253, 254] assessed the association between telomere and weight change over time in adults. In line with these studies, we found that within-subject change in waist circumference was significantly positively associated with telomere shortening. For a 1-SD increment in change in waist circumference, telomere attrition was 3.16% (95% CI: 1.30, 4.99%, *P*=0.001) higher. Results were mainly driven by women (3.19%, 95% CI: 0.72, 5.61%; *P*=0.01) and less by men (1.29%, 95% CI: -2.07, 4.54, *P*=0.44). To our knowledge only one study investigated whether within-subject change in mtDNA parallels change in obesity measures over a follow-up period.[239] No significant association was reported. In the current study, we observed a positive trend between within-subject change in mtDNA content and waist circumference in men (8.37%, *P*=0.05) and a curvilinear association in women (*P*=0.01).

In addition, we observed in **chapter 6** that abdominal obese participants and participants with increasing weight over time seemed to be more sensitive to PM exposure. The synergetic interaction between these two risk factors for chronic diseases may be due to the fact that they alter common molecular pathways, including upregulation of inflammation and oxidative stress, which are linked to mtDNA content.

Methodological implications of epidemiological research in the context of this doctoral dissertation

A major limitation of observational epidemiological studies is that they do not allow to establish causality. Compared to randomized controlled trials, they are more prone to several types of systematic errors. The first type of bias, confounding bias, occurs when an exposure of interest is strongly associated with another unknown factor that is (also) associated to the outcome. Another type of bias, information bias is present when the exposure of interest, potential confounding variables or the outcome are inaccurately assessed. The last type of

systematic error is selection bias which may produce biased exposure-outcome associations if the study population fails to mirror the population of interest. In the next paragraphs, I will provide an overview of these different types of biases regarding the chapters of this doctoral dissertation.

Residual confounding is of major concern in observational epidemiological studies. Possible sources of confounding in this doctoral dissertation include participants' genetic, demographic, socioeconomic, and lifestyle characteristics as well as meteorological factors and methodological aspects (e.g. time of blood sampling and batch effects). In all studies, we corrected for a set of potential confounders that were selected based on previous literature. As for most birth registries, we had no information on maternal nutrition, drinking, and smoking behaviour in **chapter 2**. However, a benefit of this study is that data on parental education and national origin of the mother was obtained by linkage of medical birth certificates of the SPE with data recorded by the Belgian civil birth registration. In **chapter 5** and **6**, information on a wide range of person-related characteristics (e.g. smoking status) was available. Furthermore, since we considered within-subject changes in mtDNA content and telomere length over time, the risk of confounding by participants' characteristics that do not change over time is reduced. For the cohort studies (**chapter 3-6)**, extensive self-assessment questionnaires in combination with collection of biochemical and clinical measures by trained staff provided the necessary information to correct for person-related characteristics. Since meteorological factors may be important confounders in the assessment of air pollution, we adjusted for season and/or temperature in most of the statistical models fitting short- or medium-term exposure. For long-term PM exposure (e.g. 1-year averages) the risk of confounding by meteorological factors is reduced since exposure estimates were not associated with season nor temperature. Despite the precautions regarding potential confounding, we cannot rule out residual confounding by variables that were not considered, inadequately measured, or imprecisely corrected for.

Another potential source of bias for the studies on PM exposure (**chapter 2-4, 6**) is exposure misclassification. Errors in the measurement of PM by monitoring stations and interpolation methods used to estimate individuals' PM exposure is a potential source of information bias. Furthermore, birth registries do not provide information on the residential address, therefore in **chapter 2** we could only

estimate PM exposure at the level of the municipality of residence as a proxy for individual exposure. This implicates that we did not take into account local variations in PM exposure so mothers living close to a major roadway were assigned the same PM estimate as mothers living in a rural region in the same municipality. For the cohort studies **(chapter 3-4, and 6)**, home addresses were available and linkage with air pollution data provided PM estimates at residential address. However, personal PM exposure might be quite different from the estimated outdoor PM exposure since participants may spend a large amount of time indoor or outside the direct environment of their home address, which results in exposure misclassification. Under the assumption that this is not a systematic error (i.e. non-differential misclassification), as discussed in **chapter 2**, this will lead to attenuation of effect estimates and not to a greater risk of false positive results. In **chapter 5**, the use of strict guidelines to measure participants' waist circumference, height, and weight by trained study nurses limited the risk of information bias. Errors in the outcome measurement is another potential source of information bias. Both for the molecular as well as for the fetal growth outcomes, quality assessments were performed to reduce measurement error. Despite these efforts, we cannot rule out information bias.

Since the Flemish birth registry covers (almost) all deliveries in the Flemish part of Belgium, selection bias is not of great concern in **chapter 2**. However, in the main analyses we excluded mother-newborn pairs for which marital status, parental education or national origin of the mother was missing which can result in selection bias. To rule out this type of bias, we repeated the analyses without adjusting for these covariates so including the newborns with missing data. Results remained similar suggesting that the study findings were fairly robust to the exclusion of these subjects. As for all cohort studies, for **chapter 3-6** we were dependent on the willingness of potential study candidates to participate and on the accuracy of filling in the self-assessment questionnaires, hence selection bias may be a potential source of systematic error. To maximize the completeness of the data files, subjects were re-contacted in case of missing data.

Another drawback of observational study settings is that participants are not exposed to well-specified air pollutants during specific time windows of interest. Although we assessed different exposure windows in this doctoral dissertation,

the high correlation between different time window exposures hampers the identification of the most vulnerable exposure period.

Experimental studies on animals or *in vitro* studies are not prone to the limitations discussed above, however these studies cannot be used to assess the complex mixture of ambient air pollution and interpolation of experimental findings to humans is not always meaningful.[301, 302] Epidemiological studies have the advantage to investigate humans in their natural habitat without any intervention which is not the case in an experimental study setting. Second, experiments on humans to study the effects of air pollution and excess body weight may not be consistent with ethical principles. Third, studying long-term PM effects in an experimental setting is often not feasible and since everyone is exposed to PM air pollution, no control group of non-exposed participants exists. At last, epidemiological studies are a practicable way to assess associations in susceptible population groups including pregnant women, newborns, elderly, and people with severe diseases. To this end, the ENVIR*ON*AGE birth cohort is designed to study interactions of environmental exposures with processes of ageing from early life onwards.[93] The examination at birth, including the collection of maternal blood as well as cord blood and the placenta, and follow-up examinations during childhood creates the opportunity to study both short- as well as long-term health effects during these vulnerable developmental stages. The use of birth registry data in **chapter 2** on the other hand has as major advantages the very large sample size, and large population and geographical coverage. As the samples sizes of the cohort studies of this doctoral dissertation are quite low, the power is limited. Especially for the transcriptomic studies we must say that the study findings are more prone to false discoveries due to the relatively small sample sizes and the high number of hypothesis tested. Therefore, I consider these study findings valuable in a broader context, e.g. as material for a meta-analysis, where in combination with other observational studies power to detect associations can be increased and more solid conclusions can be drawn. Moreover, our study findings can justify the performance of experimental studies, which might not receive enough funding support without the existence of observational study results.

Conclusion and future perspectives

In this doctoral dissertation, we investigated the impact of PM exposure, the fifth-ranked leading risk factor for chronic aging-related diseases,[4] on fetal growth and on potential intermediate factors driving the association between PM exposure and adverse health outcomes in both newborns and adults. Furthermore, we assessed the impact of excess body weight, another important chronic disease risk factor that gained concern in the last decades, on two molecular biomarkers of oxidative stress: telomere length and mitochondrial DNA content.

Globally, the WHO estimated that 15% to 20% of all newborns have low birth weight, representing more than 20 million newborns a year.[303] Low birth weight is an important indicator of childhood morbidity and mortality and has even health implications later in life. Elucidation of environmental factors that alter fetal growth is therefore of great importance for global public health. Consistent with previous studies, we found evidence of adverse fetal health effects at air pollution levels even below the European air quality standards. These study findings may motivate policy makers to implement stricter measures to lower air pollution and to revise the EU air quality limits in accordance to the WHO air quality guidelines. For annual PM_{10} and $PM_{2.5}$ averages this means a reduction from 40 to 20 µg/m^3 and from 25 to 10 µg/m^3.

In the ENVIR*ON*AGE birth cohort we observed an association of maternal PM exposure during the last month of pregnancy with expression of genes contributing to mitochondrial pathways in cord blood of boys. In a next step, it would be interesting to explore to which extent mtDNA content is associated with mitochondria-linked genes such as genes encoding proteins of the electron transport chain. Besides mitochondria-linked genes, we identified several genes associated with prenatal PM exposure that are implicated in air pollution-linked adverse health effects. Since ENVIR*ON*AGE is a follow-up study in which mother-children pairs are re-invited when the child is around 4 years old, further studies can link the current findings not only to indicators of adverse health issues later in life measured at birth but also in childhood.[93] E.g. olfactory receptor gene expression, which was associated with gestational PM exposure, may be linked to the neurological development at birth by using the Neonatal Behavioral assessment scale (NBAS) test and the neurocognitive capacity at the age of 4

assessed by Cambridge Neuropsychological Test Automated Battery (CANTAB) Research Suite.

In line with the findings in newborns, transcriptome analyses indicated that the mitochondrial energy-generating pathways are altered by PM exposure in adults. Moreover, we showed that mtDNA content was associated with PM exposure in two independent adult cohorts. Although this doctoral dissertation highlights the importance of mitochondria as a biosensor for environmental exposures, our study findings are only indications of PM-linked mitochondrial dysfunction. Future research should look into detail to which extent the observed gene expression changes remain stable over time and whether they can be translated to changes in protein abundance and enzyme activity.

We observed a link of obesity measures in adults with telomere length and mtDNA content, both implicated in chronic diseases. Besides this targeted approach, further studies can explore transcriptomic signatures of excess body fat as a hypotheses-generating approach to investigate potential underlying mechanisms of obesity-related chronic diseases.

Despite their limitations, the observational studies performed in this doctoral dissertation contribute to the international research investigating the impact of excess body fat and the impact of ambient PM at current exposure levels on human health. Promoting normal weight by encouraging to eat healthy and to implement physical activity in daily life, and lowering PM exposure below the WHO guidelines will help to reduce chronic disease and mortality rates worldwide.

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Dankwoord

Nu ik dit dankwoord schrijf is het einde echt in zicht! En dan komt het besef wat een leuke groep ik moet achterlaten… Ik ben men promotor Tim Nawrot dan ook heel dankbaar dat hij me overtuigde bij hem te doctoreren. Tim, je hebt je handen vol met meer dan 15 postdocs en doctoraatsstudenten en toch was het meestal jij die mij moest geruststellen als er weer eens iets fout liep. Eén keer heb je me zelfs een Chimay beloofd als het probleem zich nog een keer zou voordoen. Je hebt het talent om mensen te overtuigen, gerust te stellen en mee te nemen in je verhaal. Door jou moest ik de Rondom van Heist-op-den-Berg niet meer lezen want je hield me altijd op de hoogte ;). Moest je ooit toch willen verhuizen naar Heist-op-den-Berg, altijd welkom!

Ik wil graag de juryleden bedanken voor hun kritisch nalezen en hun bijdrage aan mijn doctoraat. Bedankt prof. dr. Theo de Kok, prof. dr. Tim De Meyer, prof. dr. Michelle Plusquin en dr. Sabine Langie!

Alle coauteurs van mijn artikels, hartelijk dank voor jullie belangrijke inbreng! Bedankt aan alle medewerkers van ENVIR*ON*AGE, FLEMENGHO, het Steunpunt Milieu en Gezondheid en het Studiecentrum voor Perinatale Epidemiologie.

En dan mijn lieve collega's… Jullie zijn de kers op de taart van men doctoraat. 5 jaar geleden begon ik hier samen met 4 van jullie. We kregen samen een plaatsje in de c107b. En een jaar later kwam er nog een vrouw bij. Dries, met 5 vrouwen op je bureau was je koptelefoon je waardevolste bezit om toch wat rust te vinden in ons kakelende kippenhok! Wat discussiëren en napraten met jou op de trein zorgde voor een mooie afsluiter van de werkdag en deed de treinrit toch iets korter lijken. Nog leuker was als jij voor taxi speelde :). Diana, je zorgt altijd voor een leuke sfeer op ons bureau! Bedankt voor alle leuke momenten en bedankt dat ik af en toe bij jou thuis kon blijven crashen. Maria, the kindest of us all, you radiate calmness and warmth to the rest of us (although it did not always work ;)). It was always nice working with you! Narjes, je drukbezette man en je 3 kindjes hebben geluk met zo'n vrolijk energiebommetje als jij! En wij ook! Met al dat lekker eten en die gastvrijheid kwamen we graag bij jou op bezoek ;). En dan de jongste van het bureau, Annette, als ik aan jou denk, denk ik aan Tuperware potjes en ochtendsportsessies! Je staat altijd voor iedereen klaar. En moest ik ooit een nieuwe vriezer nodig hebben dan weet ik wie ik moet bellen ;).

Mijn copromotoren Bianca en Karen, bedankt dat ik steeds bij jullie welkom was voor goede raad of gewoon om men hart even te luchten. Bianca, hoewel het geen liefde op het eerste gezicht was toen ik als studentje bij jou kwam aankloppen ;), hebben we nu toch veel gemeen: we zijn beiden statistici, hebben allebei onze groene vingers aangetrokken en een "makkelijke moestuin" aangelegd, zijn af en toe een tikkeltje sarcastisch en we zijn beide fan van Pascale Naesssens en Sandra Bekkari - en natuurlijk ook van transcriptoomdata ;). Het was een cadeau om met jou te mogen samenwerken! Karen Vrijens, ik denk dat ik jou bureau blindelings zou vinden. Hoeveel keer ik niet bij jou ben langs geweest? Je staat altijd klaar voor iedereen, zowel voor het werk als daarbuiten. Bedankt dat je steeds de tijd nam als ik met een vraag langskwam.

Het labowerk was een nieuwe uitdaging voor mij. Bram, Dries, Diana en Martien, bedankt voor jullie advies en hulp in het labo! Bram, ik vond het altijd inspirerend om met jou resultaten te bespreken. Michelle, bedankt dat ik altijd welkom was met al men vragen! Nelly, Martien, Kristof, Leen, Esmée en Janneke, bedankt voor alle leuke momenten op de congressen. Het was een plezier om met jullie weg te gaan! De nieuwste aanwinst van Group Nawrot: Charlotte, Evi, Yinthe, Rossella, Hanne en Katrien, bedankt voor de leuke samenwerking en de toffe teambuilding!

Nicky, Eline en Michal, ondertussen zijn we ex-collega's maar al die knotsgekke en gezellige momenten met jullie ben ik zeker nog niet vergeten! Bedankt voor al jullie advies en hulp tijdens mijn doctoraat. Harry, bedankt voor het kritisch nalezen van mijn manuscript en voor de leuke uitstap naar Averbode.

Aan al men vrienden en familie, bedankt voor jullie interesse en de nodige afleiding. Bedankt voor de leuke weekendjes weg, etentjes en de fijne uitstapjes. O.U.D.ers bedankt voor alle ELE momenten ;). Margo, je bent op de hoogte van alle ups en downs tijdens mijn doctoraat. Wat heb ik een geluk met zo'n goede vriendin!

En dan kom ik aan de personen die me het dichtst bij het hart liggen. Michiel, het is een fijn gevoel te weten dat je er altijd bent voor ons. Papa, je hebt me mee gevormd tot wie ik nu ben en me geleerd hoe belangrijk familie is in het leven. Mijn grootste motivatie, mama, je hebt me altijd alle kansen gegeven, gesteund, in me geloofd en me vertrouwen gegeven. Zonder jou had ik niet gestaan waar ik vandaag sta. Ik ben je ontzettend dankbaar voor alles wat je voor ons doet.

En dan diegene die elke hobbel in dit parcours mee gevoeld heeft. Kristof, bijna 10 jaar geleden kwam je men leven in gelopen en nog steeds heb ik het geluk om je aan men zij te hebben. Hoewel men doctoraat niet altijd van een leien dakje liep, kon je me altijd kalmeren en gerust stellen. Bedankt voor je onvoorwaardelijke steun, liefde en oneindige geduld als ik weer eens een presentatie met je wilde oefenen. Mijn publiek is nog nooit zo kritisch geweest ;). We zijn een goed team, jij en ik!

Estelle, je maakt het me heel gemakkelijk om gelukkig te zijn, te leven in het nu en alles te relativeren. Elke dag geef je me iets om naar uit te kijken en kan ik 's avonds even alles loslaten. Je laat me de mooie dingen in het leven zien.

Ellen

CURRICULUM VITAE

Ellen Winckelmans was born in Lier (Belgium) on August 29th 1988. In 2006, she graduated from secondary school at the Heilig-Hartcollege in Heist-op-den-Berg and started her study Biomedical Science at University of Leuven (KU Leuven). After obtaining her master degree (*magna cum laude*), she followed an additional Master in Biostatistics at KU Leuven during which she did her master thesis at Hasselt University. She graduated in 2013 *magna cum laude*. In the same year, she started her PhD in the research unit of Prof. dr. Nawrot at the Centre for Environmental Sciences of Hasselt University. The aim of her research was to study the effects of environmental risk factors on biomarkers of chronic aging-related health effects including mtDNA content and telomere length, and to define transcriptomic profiles within a life-course epidemiology concept. Besides teaching activities, she presented her results at several conferences including DOHad in Rotterdam and ISEE in Utrecht, Rome and Barcelona. In 2015, her paper on fetal growth and maternal exposure to particulate air pollution was chosen as Best Paper Of Year 2015 published in Environmental Research.

LIST OF PUBLICATIONS

International peer-reviewed publications

1. **Winckelmans E**, Knez J, Martens DS, Kuznetsova T, Nawrot TS. Peripheral blood telomere length and mitochondrial DNA content in relation to obesity measures: a population study. *In preparation*.

2. **Winckelmans E**, Knez J, Cox B, Kuznetsova T, Nawrot T. Peripheral blood mitochondrial DNA content in relation to long-term particulate air pollution exposure: a population study. *In preparation*.

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16. **Winckelmans E**, Cox B, Martens E, Fierens F, Nemery B, Nawrot TS. Fetal growth and maternal exposure to particulate air pollution--More marked effects at lower exposure and modification by gestational duration. *Environmental research* 2015;140:611-8.

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1. Vrijens K, Tsamou M, **Winckelmans E**, Bijnens E, Fierens F, Nawrot TS. Impact of air quality on health: identification of relevant biomarkers of exposure and effect and gene expression data from Flemish human bio-monitoring data. *The Environment, Nature and Energy Department*.

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1. **Winckelmans E**, Vrijens K, Tsamou M, Janssen BG, Saenen ND, Roels HA, Kleinjans J, Lefebvre W, Vanpoucke C, de Kok TM, Nawrot TS. Newborn sex-specific transcriptome signatures and gestational exposure to fine particles: findings from the ENVIRONAGE birth cohort. DOHaD World Congress 2017, Rotterdam, The Netherlands, 15-18 Oct 2017 (poster presentation).

2. **Winckelmans E**, Vrijens K, Tsamou M, Janssen BG, Saenen ND, Roels HA, Kleinjans J, Lefebvre W, Vanpoucke C, de Kok TM, Nawrot TS. Newborn sex-specific transcriptome signatures and gestational exposure to fine particles: findings from the ENVIRONAGE birth cohort. International Society Environmental Epidemiology (ISEE), Rome, Italy, 01-04 Sep 2016 (poster presentation).

3. **Winckelmans E**, Nawrot TS, Tsamou M, Baeyens W, De Boever P, Jennen D, de Kok TM, Vera N, Reynders H, Schoeters G, Van Larebeke N, Kleinjans J, Vrijens K. Whole genome expression analysis reveals mitochondrial responses to particulate air pollution exposure. Early Career Researchers Conference on Environmental Epidemiology (ISEE) Plaats: Utrecht, The Netherlands, 2-3 Nov 2015 (oral presentation).

4. **Winckelmans E**, Cox B, Martens E, Fierens F, Nemery B, Nawrot TS. Fetal growth and maternal exposure to particulate air pollution--More marked effects at lower exposure and modification by gestational duration. Healthy Living Conference, Maastricht, The Netherlands, 25-27 Jun 2015 (poster presentation)

5. **Winckelmans E**, Cox B, Martens E, Nemery B, Nawrot TS. Fetal growth and maternal exposure to particulate air pollution--More marked effects at lower exposure and modification by gestational duration. Young Researchers Conference on Environmental Epidemiology (ISEE), Barelona, Spain, 20-21 Oct 2014 (oral presentation)

Awards

1. Best Paper of Year 2015 published in Environmental Research: **Winckelmans** E, Cox B, Martens E, Fierens F, Nemery B, Nawrot TS. Fetal growth and maternal exposure to particulate air pollution--More marked effects at lower exposure and modification by gestational duration. *Environmental research* 2015;140:611-8.