2013•2014
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Association of particulate air pollution during pregnancy and mitochondrial DNA damage exemplified by 8-OHdG

Promotor :
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Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Content

Abbreviation list

8-OHdG	8-hydroxy-2'-deoxyguanosine
ACTB	Beta actin
ATP	Adenosine-5´-triphosphate
CI	Confidence interval
C_t	Threshold cycles
DE	Direct effect
DEP	Diesel exhaust particles
hOGG1	Human oxoguanine glycosylase 1
IE	Indirect effect
IRC	Inter-run calibrators
mtDNA	Mitochondrial DNA
MTF3212	Mitochondrial forward primer from nucleotide 3212
MTFR3319	Mitochondrial reverse primer from nucleotide 3319
MT-ND1	Mitochondrial encoded NADH dehydrogenase 1
nDNA	Nuclear DNA
NO_x	Reactive nitrogen substances
NTC	non-template controls
O_2	Oxygen
O_3	Ozone
PAH	Polycyclic aromatic hydrocarbon
PM	Particulate Matter
$PM_{0.1}$	Ultrafine particles (≤0.1 µm in diameter)
$PM_{2.5}$	Fine particles (≤2.5 µm in diameter)
PM_{10}	Coarse articles (≤10 µm in diameter)
qPCR	quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RPLP0	Acidic ribosomal phosphoprotein P0
UFP	Ultrafine particles (≤0.1 µm in diameter)
WHO	Word Heath Organization

Acknowledgments

The writing of this master thesis has been one of the most significant and enriching challenges in achieving my biomedical sciences degree. Without the support, guidance and patience of the following people, this research project would not have been completed; it is to them that I owe my deepest gratitude.

First of all, I would like to express my sincere gratitude to my daily supervisor Bram Janssen. I would like to thank him for the inspiring interest and energy he put into this project. His technical and reporting advice, were essential for the completion of this thesis and gave me new insight in the world of epidemiological research.

I would like to thank my supervisor prof. dr. Tim Nawrot who was happy to hear I wanted to go abroad for this senior project. He provided me the opportunity to perform a part of my project in a epidemiological research group in Milan, which made this project for me not just a scientific experience but an unforgettable experience of life

Furthermore, my special thanks go my external supervisor prof. dr. Valentina Bollati who also made this exchange project possible. I would like to thank for her lovely hospitality, great interest and advise during this project. It was a great pleasure to work in her research group. The warm Italian welcome and friendly atmosphere made me feel comfortable very soon, which was probably clear from my singing during my work.

In addition, I gratefully would like to thank Mirjam Hoxha who taught me the interesting techniques of the lab in Milan and helped me in completing the measurements for this project successfully . She shared with me her experienced lab skills and always knew how to calm me down in moments of panic. Working with her relevantly increased my lab experience and was every day a pleasure.

Moreover would like to thank Valeria Motta for providing me a residence room and helping me setting up in Milan; I'm thankful for all inspiring advices she gave me.

Also I'm very thankful to the people I met in Milan who made my time there a fascinating unforgettable experience. With special thanks to Roberto Campione for always helping me out of troubles in times of clumsiness and for all his crazy love and endless support. Also I would like to say thank you to Marilena Letizia for showing me the beautiful life un Milan and for the friendship, happiness and joy she still gives me.

Furthermore, I would like to thank my good friends and fellow students in Belgium for their friendship, support and motivation and for all the time they cheered me up in moments I forgot how to smile.

I also would like to thank my second examiner dr. Patrick Vandormeal for showing his interest in this project and giving me relevant advice.

Last but not least, I sincerely thank my dear family, my sister, my brother and specially my parents, for giving me all their love and endless support throughout my life. Thanks to them I am the person who really I am and want to be .

Abstract

BACKGROUND: 8-OHdG is an established biomarker for oxidative stress. Several studies emphasize the effect of particulate matter (PM) on 8-OHdG. In addition, a growing body of evidence indicates the influence of PM on mitochondrial DNA content (mtDNA), a marker for mitochondrial stress. We hypothesized that PM exposure during pregnancy results in an increase in mitochondrial 8-OHdG levels and will lead to a change in mtDNA content in maternal and cord blood.

OBJECTIVES: First, we examined 8-OHdG and mtDNA content in association with *in utero* PM_{10} exposure. Secondly, with mediation analysis we investigated intermediate mechanisms of 8-OHdG on the association of PM_{10} and mtDNA content

METHODS: We investigated maternal and cord blood from 224 mother-newborn pairs that were enrolled in the ENVIR*ON*AGE birth cohort. We determined mitochondrial 8-OHdG and mtDNA content by quantitative polymerase chain reaction (qPCR). Multi regression models were used to link 8-OHdG and mtDNA content with PM_{10} exposure over various time window during pregnancy.

RESULTS: In multivariate analysis, PM_{10} exposure during the entire pregnancy was positively associated with levels of mitochondrial 8-OHdG in maternal blood. For each IQR increment of PM_{10} exposure an increase of 23% (95% confidence interval (CI): 8.4, 39.6 p = 0.002) in 8-OHdG was observed. PM_{10} exposure during the last trimester of pregnancy was positively associated with levels of 8-OHdG (14%, 95% CI: 3.3, 28.5 p = 0.01, for each IQR increment of PM_{10}), and with levels of mtDNA content; (29.6%, 95% CI: 10.9, 51.4, p = 0.001, for each IQR increment of PM_{10}) A positive association between mtDNA content and 8-OHdG ($\beta = 0.54 \pm 0.05$, $p < 0.001$) was established. In maternal blood, 8-OHdG was estimated to mediate 42% of the positive association between PM_{10} exposure and mtDNA content during the third trimester of pregnancy and 98% during the entire pregnancy.
In umbilical cord blood. 8-OHdG levels were significantly associated with PM_{10} exposure during firth and second trimester of pregnancy with respectively an increase of 21.% (,95% CI: 5.4, 40.7, p = 0.01) and 18.5% (95% CI: 0.9, 39.0, p = 0.04) for each IQR increment of PM_{10} exposure. Moreover, we observed the same association between mtDNA content and 8-OHdG ($\beta = 0.62 \pm 0.05$, $p < 0.0001$) as found in maternal blood. No significant association was observed between mtDNA content in cord blood and PM_{10} exposure.

CONCLUSIONS: This study provides new insight into the mechanisms of altered mitochondrial function in response to *in utero* PM exposure. Mediation analysis confirmed 8-OHdG as a possible intermediate mechanism for PM_{10}-induced mtDNA alterations in maternal blood.

Samenvatting

ACHTERGROND: 8-OHdG is een veel gebruikte biomerker voor oxidatieve stress. Verschillende studies benadrukken het effect van fijn stof, oftewel particulate matter (PM), op 8-OHdG. Daarnaast, wordt het effect van PM op de mitochondriale DNA (mtDNA) inhoud, een merker voor mitochondriale stress, sterk gesuggereerd. Onze hypothese stelde dat blootstelling aan PM tijdens de zwangerschap resulteert in een stijging in de levels van mitochondriaal 8-OHdG wat zal leiden tot een verandering in de mitochondriale DNA inhoud in maternaal bloed en navelstreng bloed

DOELSTELLINGEN: Als eerste onderzochten we de associatie van 8-OHdG en mtDNA inhoud met PM_{10} blootstelling tijdens de zwangerschap. Ten tweede onderzochten we, met behulp van een mediatie analyse, het intermediaire mechanisme van 8-OHdG in de associatie tussen PM en mtDNA inhoud.

METHODE: We onderzochten het maternaal bloed en navelstreng bloed van 224 moeder-boorling paren die deel uitmaken van de ENVIR*ON*AGE geboorte cohort. 8-OHdG en mtDNA inhoud levels werden bepaald met behulp van quantitative polymerase chain reaction (qPCR). Multiregressie modellen werden gebruikt.om 8-OHdG en mtDNA inhoud te linken met PM_{10} over verschillende periodes tijdens de zwangerschap.

RESULTATEN: Mitochondriaal 8-OHdG in bloed was geassocieerd met PM10 blootstelling. Voor iedere stijging van PM_{10} blootstelling met IQR, observeerde we een stijging in 8-OHdG levels met 23% (95% confidentie interval (CI): 8.4, 39.6 p= 0.002).Daarnaast was PM_{10} blootstelling tijdens de laatste trimester van de zwangerschap positief geassocieerd met 8-OHdG levels (14%, 95%CI: 3.3-28.5 p = 0.01, elke stijgen met IQR van PM_{10}) en met mtDNA inhoud (29.6%, 95% CI: 10.9, 51.4, p = 0.001, voor elk IQR toename van PM_{10}) Bovendien werd een positieve associatie geobserveerd tussen mtDNA inhoud en 8-OHdG(β =0.54 ± 0.05, p<0.001)
In maternaal bloed, werd er een bijdrage van 42.3% van 8-OHdG geschat voor de mediatie tussen mtDNA inhoud en PM_{10} blootstelling tijdens de trimester van de zwangerschap. Voor de volledige zwangerschap was dit 98%.
In navelstreng bloed, werden 8-OHdG levels significant geassocieerd met PM_{10} concentraties tijdens de eerste (21.% ,95% CI: 5.4, 40.7, p = 0.01, elke stijgen met IQR van PM_{10}) en tweede trimester (18.5% 95% CI: 0.9, 39.0, p = 0.04, voor elke IQR stijging van PM_{10}) van de zwangerschap. Bovendien vonden we ook voor navelstrengbloed een associatie tussen mtDNA inhoud en 8-OHdG levels (β =0.62±0.05, p<0.0001).

CONCLUSIE: Deze studies geven nieuwe inzichten in het mechanisme van een veranderde mitochondriale functie als respons op PM blootstelling tijdens de zwangerschap. Mediatie analyse bevestigt dat schade in mtDNA een intermediair mechanisme is voor de PM_{10} geïnduceerde veranderingen in mtDNA inhoud in maternaal bloed

List of figures

List of tables

1 Introduction

Despite the fact that air pollution levels declined the last few decades, it still poses a problem for the environment and human health. Air pollution can be subdivided in particulate matter (PM), ozone (O_3), reactive nitrogen substances (NO_x) and some organic compounds. The part of air pollution which is most relevant to human health is PM, as it contains small toxic particles that are able to penetrate deep into sensitive regions of the respiratory system and give rise to severe health problems. [1-3]

1.1 Origins of ambient particulate matter

PM is the general term used for a mixture of particles (solid and liquid) suspended in the air, with a wide range of chemical compositions and sizes. PM originates from natural as well as anthropogenic sources. Natural sources include pollen, naturally suspended dust, sea salt, and volcanic ash. Anthropogenic sources include fuel combustion for vehicles, incineration, domestic heating for households and fuel combustion in thermal power generation. In cities, relevant local sources include vehicle exhausts, road dust resuspension, and the burning of wood, fuel or coal for domestic heating. These are all low emitters (below 20 meters), resulting in significant impacts on the concentration levels close to ground.[1, 4]

Furthermore, PM can be categorized as either primary or secondary particles. Primary PM is directly emitted in the atmosphere (e.g. from chimneys) while secondary PM is formed in the atmosphere from the oxidation and transformation of primary gaseous emissions. These gaseous emissions that contribute to the formation of PM are also known as precursor gases. The most important precursor gases for secondary particles are NO_X, ammonia, sulphur dioxide, and volatile organic compounds, a class of chemical compounds whose molecules contain carbon. The composition of PM is location-specific due to a variety of factors including chemical, physical and metrological conditions that determine the formation of PM. Generally, the previously mentioned main chemical components of PM account for about 70% or more of the mass of PM_{10} and $PM_{2.5}$. The remaining 30 % is supposed to be made up of water. [1, 4]

Relevant to human health, PM can be classified on their size and is expressed in micrometres (µm) (Figure 1). The largest particles of concern measure 10 µm in diameter or smaller (PM_{10}) and are termed as coarse particles which consist of mainly sea salt, wind-blow dust, pollen, mold, spores and endotoxins. However, the group of particles of most concern to human health is $PM_{2.5}$ (2.5 µm in diameter or smaller), also known as fine particles. These fine particles include combustion particles, organic compounds, sulphates, nitrates, ammonium and toxic metals. Within this group, particles with an aerodynamic diameter smaller than 0.1 µm are categorized as ultrafine particles (UFP or $PM_{0.1}$) and are potentially the most dangerous due to their small size, large surface area, high content of redox cycling organic chemicals. Noteworthy to mention is that fine and ultrafine fractions represent more than 50% of PM_{10} composition. The smaller the particle size, the higher the ability of the toxic particles to penetrate deep into the lungs and enter blood stream, leading to health problems. [1, 5, 6]

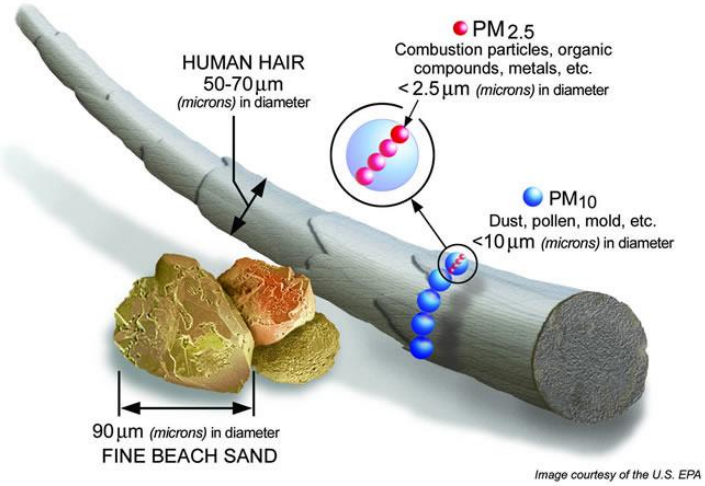

Figure 1 Different sizes, components and sources of particulate matter. PM_{10} is known as coarse particles which includes mainly sea salt, wind-blow dust, pollen, mold, spores and endotoxins. $PM_{2.5}$ are termed as fine particles and mainly include combustion particles, organic compounds and toxic metals [5]

1.2 Heath effects of particulate matter

During the last years, increasing epidemiological evidence attributes severe health outcomes to PM exposure. Recent long−term studies establish associations between PM exposure and mortality at levels strong below the current annual WHO air quality guideline level for PM_{10} (i.e. not more than 35 days per year with a daily average concentration exceeding 50 µg/m^3). Moreover, scientific evidence does not indicate a threshold of exposure to PM of which no adverse health effects would be anticipated [1, 7-9]. The current levels of PM exposure experienced by most urban and rural populations have harmful effects on human health. Chronic exposure to PM contributes to the risk of developing respiratory and cardiovascular diseases, as well as lung cancer. [8-11]

The health effects of PM are caused through their inhalation and penetration into the lungs and blood stream, giving rise to adverse effects in the respiratory, cardiovascular, immune, and neural systems. Both chemical and physical interactions between PM and lung tissues can result in irritation, inflammation and damage. Ultrafine particles (with diameters of 0.1 micrometres or less) can even penetrate into the brain by passing the blood-brain barrier. [12, 13]

1.2.1 Susceptible population groups

Although a lot studies focus on the effects of air pollution on mortality and cardiorespiratory morbidity in adult life, it is highlighted that some age groups are more susceptible than others. In addition to the pronounced effects observed in the elderly population, studies in children suggest that also the opposed end of the age spectrum is more vulnerable to air pollution compared to the general population[14]. Emerging evidence indicates that air pollution is associated with elevated risk of adverse pregnancy outcomes [15-17].

Taken together, foetuses and children, the elderly, and persons with pre-existing diseases are populations with different biological capacity to respond to air pollution exposure leading to a higher susceptibility for its detrimental health effects. Differential risks for women and men are less clear,

although growing evidence on reproductive and birth outcomes point to pregnant women as a susceptible group that has not yet been explicitly identified[18]. Pregnancy has been associated with maternal systemic inflammation [19], which probably impairs the biological response to particulate air pollution and increases the risk to the negative effects of PM. Due to their relatively small size, inhaled PM can enter the bloodstream and escape phagocytosis. This may lead to systemic effects of PM throughout the body. Oxidative stress and inflammation responses occur in the lungs and other organs, including the placenta, thereby increasing the susceptibility of the mother and possibly also the foetus [20, 21]. Consequently, this mechanism can explain the established association between PM exposure during pregnancy and adverse health effects [22].Taking all these considerations into account, the interest increases to further investigate the effect of PM exposure during pregnancy on foetal and maternal health outcomes.

1.3 Molecular effects of particulate matter

Although the underlying mechanism of adverse health effects and PM are not completely understood, several mechanism have been suggested to play an important role including inflammation, stimulation of capsain/irritant receptors, endotoxin effects, covalent modification of cellular components, endotoxin effects, pro-coagulant effects, and reactive oxygen species (ROS) production. [6, 23]

1.3.1 Oxidative stress

Among several proposed underlying mechanisms driving the health effects of PM, the generation of oxidative stress with ROS production has received most attention. Moreover, emerging data suggest a key role for oxidative stress in the underlying mechanism linking PM exposure to pulmonary and cardiovascular diseases. [6, 24-26]

ROS are chemically reactive molecules and are formed as a natural by-product of the normal metabolism of oxygen (O_2). ROS are involved in various enzyme-catalysed O_2 reactions and play an important role in cell signalling and homeostasis. However, when ROS levels increase dramatically and the pro-antioxidant balance is disrupted, e.g. during times of environmental stress, ROS are able to induce deleterious effects. This condition is known as oxidative stress.

In normal aerobic metabolism, oxidative phosphorylation takes place in the mitochondrion and leads to the production of adenosine-5´-triphosphate (ATP). During oxidative phosphorylation, O_2 serves as an electron acceptor, which under normal coupling conditions requires four-electrons to form H_2O. However, several kinds ROS are formed during this reaction (Firgure 2)

ROS are highly reactive with DNA, lipids and proteins and to prevent their injurious effects, ROS are neutralized by a variety elaborated defence system of antioxidants. This system includes several antioxidant enzymes, proteins, and low molecular weight scavengers. In conditions of abundant ROS production, which may be the consequence of PM exposure, the antioxidant defences may be overwhelmed, leading to a state of cellular oxidative stress.[25, 27, 28]

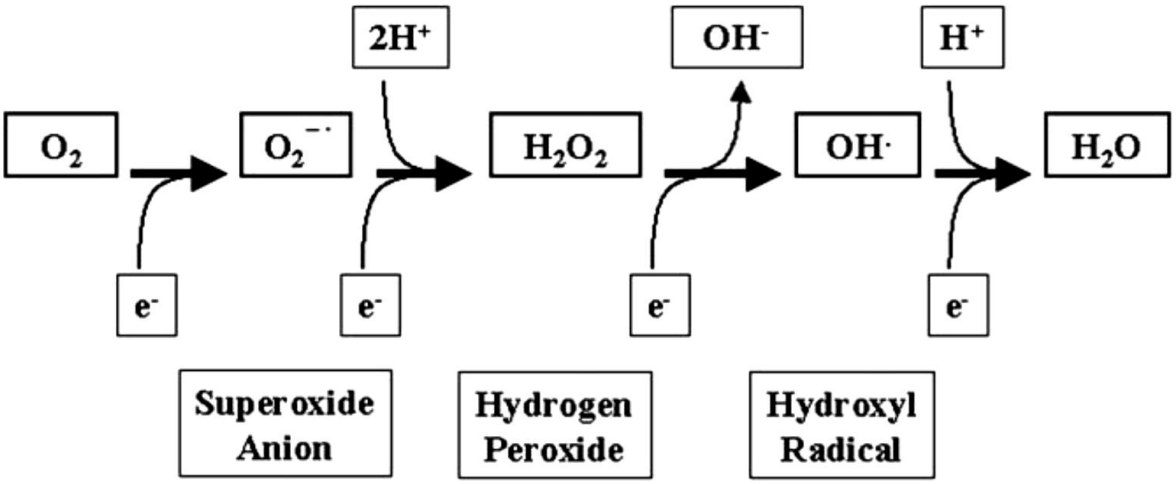

Figure 2 ROS production during oxidative phosphorylation. In this process, O_2 receives four electrons to form H_2O. Different forms of ROS can be formed during this cascade when O_2 accepts an incomplete amount of electrons. The addition of only one electron results in the formation of the superoxide radical ($O_2^{-\cdot}$). The next addition of an electron leads to the formation of hydrogen peroxide (H_2O_2), and thereafter the hydroxyl radical ($OH^{\cdot}$). Other types of ROS include singlet oxygen ($O^{\cdot}$), reactive anions that contain oxygen atoms (e.g., OCOO-, peroxynitrite), molecules containing oxygen atoms that can produce free radicals (e.g., HOCl), and ozone**.**

1.3.2 Hierarchical oxidative stress response model as a mechanistic platform of particulate matter induced health effects

The important role of ROS in PM-induced adverse health effects has been highlighted using diesel exhaust particles (DEP) as a oxidative stress model of ambient coarse and fine PM. As a result, a hierarchical cellular response model has been developed to explain the role of oxidative stress in mediating the biological effect of PM [25, 29, 30]. This 3-tier model (Figure 3) indicates that low levels of oxidative stress induce protective effects and high levels of oxidative stress induce more damaging effects. The protective effects (Tier 1) are induced by several transcription factors leading to transcriptional activation of > 200 antioxidant and detoxification enzymes . Due to the protective Tier 1 response, particle-induced ROS production does not automatically lead to adverse biological outcomes. In case these protective responses fail to provide adequate protection, a further increase in ROS production can result in pro-inflammatory (Tier 2) and cytotoxic (Tier 3) effects. Pro-inflammatory effects are mediated by the redox-sensitive cascades that are responsible for the expression of cytokines, chemokines , and adhesion molecules, many of which are involved in the inflammatory process of the lung. Mitochondria play an important role in Tier 3 (cytotoxic effects, a.k.a toxic oxidative stress). Mitochondria try to encounter the overproduction of ROS capable of releasing pro-apoptotic factors that induce apoptosis [31, 32]. It is important to note that the toxicity of PM may be related, not only to its aerodynamic diameter and alveolar deposition capacity, but also to PM composition, since different components of PM can contribute to inflammation and oxidative stress. Taken together, the hierarchical cellular oxidative stress model provides a mechanistic platform of PM–induced oxidative stress and highlights an important role of mitochondria in the response to oxidative stress.[25]

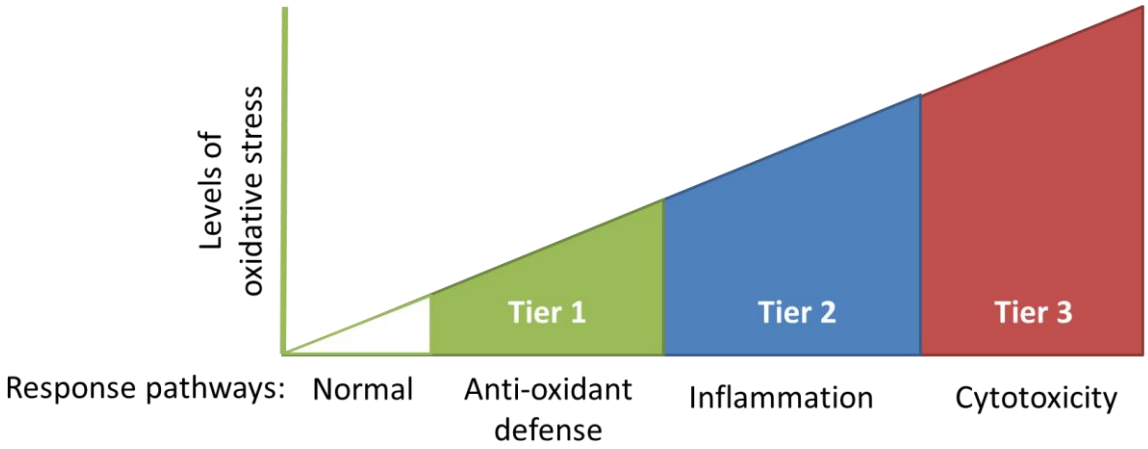

Figure 3 Hierarchical oxidative stress responses model. At a low level of oxidative stress (Tier 1), antioxidant enzymes are induced to maintain cellular redox homeostasis. At an intermediate level of oxidative stress (Tier 2), pro-inflammatory responses are induced. At a high level of oxidative stress (Tier 3), perturbation of the mitochondrial permeability transition pore and disruption of electron transfer result in cellular apoptosis or necrosis. The hierarchical cellular oxidative stress model provides a mechanistic platform against which we try to understand how PM generates adverse health effects. [25]

1.4 Importance and vulnerability of mitochondria

As mentioned in section 1.3.1, cellular energy provision is mainly obtained by the production of ATP via oxidative phosphorylation of O_2 in the electron transport chain in the mitochondria. Mitochondria are essential providers of cellular energy and, consequently, the integrity of mitochondrial function is fundamental to cell life. Moreover, mitochondria are the main intracellular source and target of ROS that are continually generated as by-products of mitochondrial respiration in the electron transport chain. Low levels of ROS generated from the respiratory metabolism is proposed to take part in the signalling from mitochondria to the nucleus. Several structural characteristics of mitochondria and the mitochondrial genome enable them to sense and respond to extracellular and intracellular signals or stresses in order to maintain the life of the cell. It has been established that mitochondrial respiratory function declines with age, and that defects in the electron transport chain increase the production of free radicals and ROS in mitochondria. Considering the aforementioned hierarchical oxidative stress response model (Figure 3), ROS within a certain concentration range may induce stress responses of the cell by altering the expression of a number of genes in order to uphold energy metabolism to rescue the cell. Nevertheless, beyond this threshold, ROS may induce apoptosis trough induction of mitochondrial membrane permeability transition and release of cytochrome c.[33, 34]

1.4.1 Mitochondrial DNA content as a marker of mitochondrial damage

One of the compensating stress responses is the increase in mitochondrial abundance and copy number. Each cell contains approximately 200–2,000 mitochondria, each carrying 2–10 copies of circular mitochondrial DNA (mtDNA). Interestingly, the size and number of mitochondria is correlated with mtDNA content, and have been shown to change not only under different energy demand, but also by different physiological or environmental conditions. Contrary to human nuclear DNA (nDNA), mtDNA lacks the protective chromatin structure, histones and introns. Moreover, the mtDNA repair mechanisms work less efficiently compared to the one of nDNA. Consequently, these differences and additionally the close proximity to the electron transport chain explain the higher probability of

mtDNA damage compared to nDNA damage. Cells challenged with ROS have been shown to increase their mitochondrial abundance and synthesize more copies of their mtDNA in order to compensate for damage and encounter the increased respiratory demand which is required for ROS clearance [34]. In turn, the increased amount of mitochondria in these cells lead to an additional increase in the production of ROS and, therefore, intensify the oxidative damage to mitochondria and other intracellular constituents including DNA, RNA, proteins, and lipids. Once beyond the threshold, ROS may elicit apoptosis which may result in a decrease in mtDNA content [35]. These assumptions can be linked to the earlier mentioned Trier model (Figure 3) which indicated that oxidative stress can only be compensated below certain concentrations of ROS.

Accordingly, mtDNA content is an established marker of mitochondrial damage and malfunctioning and may represent an important biological effect in the pathway linking PM to adverse health effects. Several studies have investigated the effect of PM, in form of environmental factors as well as personal exposures (smoking), on mtDNA content (Supplemental Table S1). In these studies both an increase and decrease of mtDNA content were related to PM exposure which may refer to the oxidative stress responses that are level dependent compensatory mechanisms t(Tier model, section 1.3.2).

1.4.2 8-OHdG as a marker of oxidative stress in genomic and mitochondrial DNA

As mentioned before, ROS are highly reactive and able to induce DNA damage. Among the different types of ROS-induced DNA damage, the oxidation of guanine and thus the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG is) the most common one. Therefore, 8-OHdG is frequently used as a biomarker for oxidative stress [36]. Because this biomarker can cross the cell membrane, levels of 8-OHdG in urine and blood serum are often used as assessment for oxidative stress [37]. In many studies, 8-OHdG has widely been used, not only as a biomarker for the measurement of oxidative DNA damage, but also as a risk factor for aging and many diseases associated with oxidative diseases including cancer [38].

Increased 8-OHdG levels in urine and blood have been associated with exposure to PM in healthy male subjects in several occupational studies. Nevertheless, most of the occupational studies had PM exposure levels that greatly exceed the US Environmental Protection Agency (EPA) particulate air pollution limits. In order to assess the association between 8-OHdG and PM exposure levels similar to the outdoor environment experienced by the general population, several non-occupational studies human subject studies were reviewed by Benson et al.(2013) [39]. An overview of the studies is shown in Supplemental Table S2. Overall, most studies found a positive association between exposure to particulate air pollution and 8-OHdG levels in members of the general public including children, adults and military veterans. In addition, several experimental studies show increased levels of 8-OHdG in response to DEP exposure that is used as an oxidative stress model of ambient coarse and fine PM. These studies confirm the usage of 8-OHdG as a biomarker for oxidative stress [40, 41].

Besides the levels of mtDNA content, the oxidative mutation rate of mtDNA can serve as a marker for endogenous oxidative stress linking PM exposure to its adverse health effects. As mentioned before, in addition to ROS formation by external factors including PM, ROS are also produced as a by-product of mitochondrial respiration in the electron transport chain which makes mtDNA more prone to oxidative damage. However until only a few studies used the levels of 8-OHdG in mitochondrial as a

marker for oxidative stress and thus this method of determining 8-OHdG levels is relatively new. [42, 43].This is first project using 8-OHdG in mtDNA as a biomarker in an epidemiological l approach

1.5 Aim of the ENVIRONAGE birth cohort project

The present study is a part of the large ongoing birth cohort called ENVIR*ON*AGE (the acronym emphasizes the ENVIR*ON*mental influence on the AGEing process). The process of aging is known as the primary risk factor for the development of chronic conditions including cardiovascular and respiratory diseases and is known to be influenced by environmental factors. [44]

Because it is suggested that these aging complications in adult life already can originate from *in utero* life [45], this study suggests that environmental factors and lifestyle factors may induce molecular changes in the aging process which alter *in utero* development and may lead to adverse birth effects that form the origin of diseases arising in adult life.
However, to consolidate these effects to the foetus, we should first investigate the pregnant mothers. The impact of PM pollution on pregnant women itself has been poorly investigated, and it would be of great interest to give more insights in how the effects of *in utero* PM exposure influences both the health of the mother and unborn child

1.5.1 Hypothesis

In the present project, we focus on PM, which is the type of air pollution most relevant to human health. The underlying biological mechanisms which could explain the association between PM exposure and adverse health problems are poorly understood. However, the formation of ROS and induction of inflammation due to PM, and its associated metal compounds, is thought to be of importance because ROS are able to induce damage to DNA, lipids and proteins. We hypothesize that PM exposure during pregnancy will induce systemic oxidative stress, exemplified by 8-OHdG levels in maternal blood and will lead to a change in mtDNA content in maternal and cord blood.

The objectives of this study are threefold; 1/ We will examine the association between PM exposure and 8-OHdG levels measured in mitochondria of maternal blood. 2/ We will examine the association between PM exposure and mtDNA content in maternal blood. 3/ We will investigate whether the effects we observe in maternal blood are translated to the foetus by examining 8-OHdG levels and mtDNA content in cord blood .

2 Materials and methods

2.1 Study population and data collection

Mother-newborn pairs were recruited from the Hospital in South-Limburg (ZOL; Genk) in Belgium since 2010. Up until now the ENVIR*ON*AGE birth cohort consists of more than 660 mother-infant pairs.

Participating mothers had to complete a study questionnaire to provide detailed information on age, socioeconomic status, ethnicity, smoking status, place of residence, pre-gestational body mass index (BMI), and parity. Socioeconomic status was coded and condensed into a scale with scores ranging from 0 to 2 based on mother's education. In particular, maternal education was coded as low (no diploma or primary school), middle (high school) or high (college or university degree). Current smokers were specified as having smoked before and during pregnancy. Before-smokers were specified as those who had quit before pregnancy, and never-smokers had never smoked. Immediately after delivery perinatal parameters such as newborn's sex, birth date, birth weight and length, gestational age (range, 35–42 weeks), Apgar score, and ultrasonographic data were collected The present study was conducted according to the principles outlined in the Helsinki Declaration (World Medical Association 2008) for investigation of human subjects. Written informed consent is provided by all study participants in accordance with procedures approved by the Ethical Committee of Hasselt University and ZOL Genk.

2.1.1 Exposure assessment

In order to estimate levels of ambient PM exposure the spatial interpolation Kriging method was applied. This validated method allows us to interpolate the regional background levels of ambient PM for each mother's residential address based on data obtained from satellite images in combination with monitoring stations. This model provides interpolated $PM_{2,5}$ values from the Belgian telemetric air quality networks in 4 × 4 km grids, and for PM_{10} values in a higher resolution of 10x10 grids

In order to explore potentially critical exposure periods during pregnancy, individual means PM concentrations were calculated for various periods including of the three trimesters of pregnancy, with trimesters being defined as: 1–13 weeks (trimester 1), 14–26 weeks (trimester 2) and 27 weeks to delivery (trimester 3). The date of conception was estimated based on ultrasound data.

2.2 Sample collection and processing

Immediately after delivery samples of maternal and umbilical cord blood were collected in Vacutainer® Plus Plastic K2EDTA Tubes (BD, Franklin Lakes, NJ, USA).Samples were centrifuged at 3,200 rpm for 15 min to retrieve buffy coats and instantly frozen, first at –20°C and afterwards at –80°C.

2.2.1 DNA extraction

Genomic DNA was extracted from the white blood cell containing both nuclear and mitochondrial DNA. From white blood cells buffy coat DNA was purified in the using the QIAamp® DNA minikit following the manufactured instructions. Briefly, this extraction kit uses a lyses buffer, a proteinase and a RNase to induce cell lyses and remove proteins and RNA. Afterwards ethanol and the silica-membrane spin-columns and buffers from the kit were used to purify and collect the DNA.
The concentration of extracted DNA was measured at 260 nm with the Nanodrop spectrophotometer (ND-1000; Isogen Life Science, De Meern, the Netherlands). Both DNA yield (nanograms per microliter) and purity ratios (A260/280 and A260/230) were determined. Extracted DNA was stored at –20°C until further use. Mean DNA yield was 167.6 ng/µl with purity values of 1.9 for A260/280 ratio and 2.1 for A260/230 ratio. Extracted DNA was stored at -20° for further use

2.3 Determination of the 8-OHdG levels in mitochondria

8-OHdG measurements were carried out at the highly recognized research centre of Molecular Epidemiology and Genetics at the University of Milan. The Laboratory has experience in measuring 8-OHdG levels and recent work of this research group shows a good reproducibility of these measurements.[42]
8-OHdG in mtDNA was measured using quantitative real-time polymerase chain reaction (qPCR) based on the following principle. The enzyme human oxoguanine glycosylase 1 (hOGG1) can recognize 8-OHdG and catalyse its removal through cleavage of the phosphodiester bond. Although 8-OHdG does not distort the structure of DNA, digestion of the 8-OHdG by hOGG1 can break the DNA template and block the amplification of this region [46]. Consequently, as illustrated in Figure 4, the difference in amplification efficiency (ΔC_t) between a treated(C_{t1}) and non-treated(C_{t2}) aliquot of the DNA sample gives an indication for the amount of 8-OHdG present in the DNA region [43, 47].

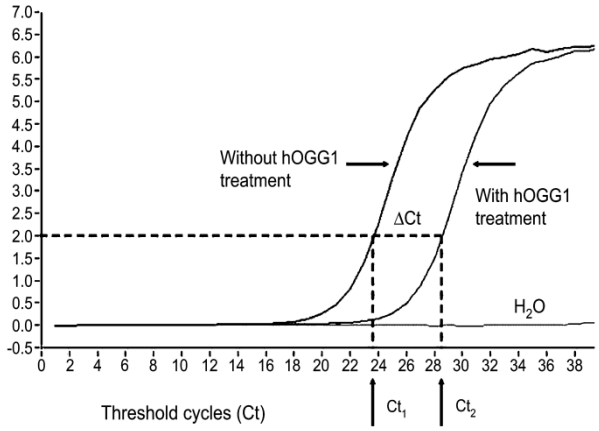

Figure 4 Determination of the degree of oxidative mtDNA damage. As applied in previous research[43] the degree of 8-OHdG in mtDNA can be quantified by the difference in amplification efficiently (ΔC_t) between aliquots of a sample treated with hOGG1 (C_{t2})and without (C_{t1}) the enzyme hOGG1 (human oxoguanine glycosylase). In case the sample contains 8-OHdG lesion the treatment with hOGG1 will reduce the PCR efficiency and thus increase the Ct value (from C_{t1} up to C_{t2})

To create ideal condition for the enzymatic reaction of hOGG1, the extracted genomic DNA was diluted to a concentration of 6.25 ng/µL in RNase free water. One aliquot (4µl) of the samples was treated with 11µl treatment mix containing hOGG1 while a second aliquot was treated with the same mix containing H_2O instead of hOGG1. The mix consisted of RNase free water (8.7µl/reaction), Buffer NE 10X (1.5µl/reaction), BSA 100X (0.15 µl/reaction), and hOGG1 or RNase free water (0.625µl / reaction) for treatment mix and non-treatment mix respectively. All treated and non-treated samples were each loaded in a 'treated' and 'non-treated' 96-Well Reaction plate. After an incubation of these plates during 1hour at 37°C the samples were put on ice to block the enzymatic reaction.

Afterwards samples were diluted 1:4 in RNase free water for the qPCR runs. PCR reactions were set up by aliquoting 6 µL master mix into each well of and 4 µL of each experimental DNA sample, resulting in a final volume of 10 µL per reaction. PCR master mix consisted of TaqMan® Fast Advanced Master Mix (Applied Biosystems; 5 µL/reaction), forward (0.5 µL/reaction) and reverse (0.5 µL/reaction) Primers *MTF3212/R3319* (mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319,Table 1) were used for the amplification of mtDNA. Aliquoting of the samples for the treatment step as well as for the preparation of real-time-PCR plate was performed automatically by MICROLAB® STARlet. The use of the workstation increases accuracy and repeatability while providing chain of custody with pipette condition monitoring and recording. Runs were performed in a MicroAmp® Fast Optical 384-Well Reaction Plate compatible with the 7900HT Fast Real-Time PCR System. In each plate we took along two non-template (NTC) controls. To be able to correct for plate variability four inter-run calibrators (IRC) were included in each PCR plate. The thermal cycling profile started with of 10 sec at 95°C for activation of the DNA-polymerase, followed by 35 cycles of 15 sec at 95°C plus denaturation and of 1 min at 60°C for annealing/extension. Afterward a dissociation step was performed (15 sec at 95°C, 15 sec at 60°C, 15 sec at 95°C) to control for the specificity and absence of primer dimers. To increase the accuracy of the measurements all samples were loaded in triplicate.

After each run raw data were collected and we corrected the C_t values for inter-run variation using qBase software (Biogazelle, Zwijnaarde, Belgium) (50). Afterwards, we calculated the differences between C_ts to use in statistical analysis.

2.4 Determination of the mitochondrial DNA content

mtDNA content was measured in leukocytes of maternal and cord blood by determining the ratio of two mitochondrial gene copy numbers [*MTF3212/R3319* (mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319) and *MT-ND1* (mitochondrial encoded NADH dehydrogenase 1)] to two single-copy nuclear control genes [*RPLP0* (acidic ribosomal phosphoprotein P0) and *ACTB* (beta actin)]. (Table 1) The quantities of this genes were determined by using qPCR assay. In advance of the PRC runs all extracted genomic DNA samples were diluted to a final concentration of 5 ng/µL in RNase free water. Each run was performed by the 7900HT Fast Real-Time PCR System (Applied Biosystems). PCR reactions were set up by aliquoting 7.5 µL master mix into each well of a MicroAmp® Fast Optical 384-Well Reaction Plate followed by 2.5 µL of each experimental DNA sample, for a final volume of 10 µL per reaction. The master mix included Fast SYBR® Green I dye 2× (Applied Biosystems; 5 µL/reaction), forward (0.3 µL/reaction) and reverse (0.3 µL/reaction) primer and RNase free water (1.9 µL/reaction). Primer sequences were diluted to a final concentration of 300 nM in the master mix. In each plate we took along two non-template (NTC) controls. To adjust for plate variability, six IRCs were included in each PCR plate. The thermal cycling profile started with 20 sec at 95°C for activation of the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 sec at 95°C for denaturation and 20 sec at 60°C for annealing/extension. Likewise, amplification specificity and absence of primer dimers was confirmed by melting curve analysis at the end of each run (15 sec at 95°C, 15 sec at 60°C, 15 sec at 95°C).

At the end of each run, raw data were collected and processed. C_T (cycle threshold)–values of the two mitochondrial genes were normalized relative to the two nuclear reference genes using the qBase software [48]. We used qbase software to correct for inter-run differences.

Table 1 Mitochondrial and nuclear primer sequence information based upon Assembly GRCh37/hg19 of the UCSC genome browser

Gene symbol	Chr	Amplicon start-end	Primer set (Forward / Reverse)	Amplicon (bp)	Primer efficiency (%)
MTF3212/R3319	M	3213-3320	F:5'-CACCCAAGAACAGGGTTTGT-3' R:5'-TTAACAACATACCCATGGCCA-3'	108	96.3
MT-ND1	M	3314-3428	F:5'-ATGGCCAACCTCCTACTCCT-3' R:5'-AAAGGCCCCAACGTTGTAG-3'	115	99.3
RPLP0	12	120636904-120636988	F:5'-CCCAATTGTCCCCTTACCT-3' R:5'-GAACACAAAGCCCACATTCC-3'	85	100.7
ACTB	7	5567833-5567934	F:5'-ACTCTTCCAGCCTTCCTTCC-3' R:5'-TGTGGAAGCTAAGTCCTGCC-3'	102	96.8

Mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (*MTF3212/R3319*); Mitochondrial encoded NADH dehydrogenase 1 (*MT-ND1*); Acidic ribosomal phosphoprotein P0 (*RPLP0*); Beta actin (*ACTB*).

2.5 Statistical analysis

In order to perform statistical analysis and database management the SAS V.9.3 (SAS Institute Inc., Cary, North, Carolina, USA) was used. Spearman and Pearson correlation coefficients and multiple linear regression were used to assess the association of mtDNA content and mtDNA 8-OHdG in maternal bloods with PM_{10} exposure.

Covariates considered for entry in the model ($p \leq 0.10$) using single linear regression included maternal age, gestational age, maternal education, smoking status, alcohol consumption and season of delivery. Maternal age, smoking status, gestational age and maternal education were forced into the model regardless of the *p*-value.

Finally, a mediation analysis was performed to explore the role of mitochondrial 8-OHdG as a mediator of the association between PM exposure and mtDNA content . This approach decomposes the total observed effect of PM pollution on mtDNA content into a direct effect of exposure and an indirect effect (IE) of PM that acts via the mediator of interest, which in this case was mitochondrial 8-OHdG) [49].

Mediation analysis requires a significant relation of the outcome to the exposure, a significant relation of the outcome to the mediator and a significant relation of the mediator to the exposure; as potential mediator. The IE of 8-OHdG was assessed with the causal step approach[50].

3 Results

3.1 Population characteristics and exposure levels

In the current project, 224 pregnant women were included with an average age of 29 ± SD 4.8 years (range 17 - 44 years). Lifestyle factors were collected by means of a self-reported questionnaire and are displayed in Table 2. Mean body mass index (BMI) before delivery of participating mothers averaged 24.2 (± 4.8) kg/m^2. 128 participants (37.1%) were high educated while 24 (10.7%) participants had a low education. 31 mothers (13.8%) reported to have smoked during pregnancy. 39 women (17.4%) had smoked before pregnancy and most of the mothers (*n* = 154, 68.8%) never smokes cigarettes. Most mothers (*n* = 178, 79.5%) did not consume alcohol during their pregnancy. 19.2% (*n* = 43) of the population reported to have consumed never more than one alcoholic drink a day, while 3 participants (1.3%) reported to have consumed more than one alcoholic drink a day. 46.4% (*n* = 104) of the newborns were males. Gestational age averaged 39.3 (± 1.2) weeks and delivery took place equally spread over all seasons of the year.

Table 2 Characteristics of mother-newborn pairs (*n* = 224)

Maternal characteristics	**Mean ± SD or range and number (%)**
Age, y	29 ± 4.8
Pre-gestational BMI, kg/m^2	24.3 ± 4.8
Gestational age, weeks	39.3 ± 1.2
Maternal education	
Low	24 (10.7)
Middle	72 (32.2)
High	128 (57.1)
Smoking	
Never-smoker	154 (68.8)
Past-smoker	39 (17.4)
Smoker	31 (13.8)
Alcohol	
No	178 (79.5)
< 1 drink / day	43 (19.2)
> 1 drink/day	3 (1.3)
Newborn's gender	
Male	104 (46.4)
Female	120 (53.6)
Season of delivery	
Fall	62 (27.7)
Winter	37 (16.5)
Spring	66 (29.5)
Summer	59 (26.3)

Table 3 displays the mean ambient exposure to PM_{10} during pregnancy. PM_{10} exposure averaged 20.9 ± 5.4 μg/m^3 with an interquartile range (IQR) of 7 μg/m^3 for trimester 1, 21.5 ± 4.8 μg/m^3, IQR: 7.4 μg/m^3 for trimester 2, and 22.3 ± 5.6 μg/m^3, IQR: 8.5 μg/m^3 for trimester 3. During the entire pregnancy, mothers were exposed to an average PM_{10} concentration of 21.5 ± 2.2, IQR: 3 μg/m^3.

Table 3 Exposure characteristics (*n* = 224).

Time window PM_{10}, μg/m^3	Mean ± SD	25th Percentile	75th percentile	IQR
Trimester 1 (1-13w)	20.9 ± 5.4	17.6	24.6	7
Trimester 2 (14-26w)	21.5 ± 4.8	17.5	24.9	7.4
Trimester 3 (27w-delivery)	22.3 ± 5.6	17.3	25.8	8.5
Last month	22.7 ± 7.9	16.1	28.2	12.1
Entire pregnancy	21.5 ± 2.2	20.2	23.2	3

3.2 Predictors of mitochondrial DNA content and mitochondrial 8-OHdG in maternal blood

First, we identified potential predictors of mtDNA content and 8-OHdG in maternal blood that may influence the association between our studied outcomes and PM_{10} exposure. Covariates considered for entry in the model are reported in **Table 4** and included maternal age (years), pre-gestational BMI, (kg/m^3), gestational age (weeks), smoking status (never, before, current), season of delivery (fall, winter, spring, summer), maternal education (low, middle, high), and alcohol consumption during pregnancy (never, never more than 1 consumption a day, more than one consumption a day). Levels of mtDNA content where positively associated with maternal smoking during pregnancy (β = 0.106 ± 0.04, $p < 0.01$) compared to non-smokers. An inverse association was observed between levels of mtDNA content and alcohol consumption during pregnancy (β = -0.063 ± 0.04, $p < 0.09$) Levels of 8-OHdG were significantly associated with smoking before pregnancy (β = 0.098 ± 0.04, p = 0.02) compared with non-smokers. A significant decrease in 8-OHdG is observed from deliveries during spring (β = -0.090 ± 0.04, p < 0.04) and summer (β = -0.088 ± 0.04, p < 0.4) compared to deliveries during fall (reference). Variables being significant associated with 8-OHdG levels or mtDNA content ($p \leq 0.10$) were entered as covariates in the statistical model. Although maternal age, maternal education, gestational age and season of delivery were not statistically associated with either 8-OHdG or mtDNA content, we forced these variables into the multiple regression model regardless of the *p*-value, together with, smoking status and alcohol consumption.

Table 4 Predictors of 8-OHdG and mtDNA content in mother-newborn pairs (*n* = 224)

	8-OHdG			mtDNA content		
Variables	**β**	**SE**	***p*-value**	**β**	**SE**	***p*-value**
Age, y	0.001	0.003	0.74	-0.004	0.003	0.15
Pre-gestational BMI, (kg/m^2)	0.001	0.003	0.80	0.001	0.003	0.86
Gestational age	0.009	0.01	0.47	0.01	0.01	0.26
Maternal education						
Low	Ref			Ref		
Middle	0.05	0.06	0.32	0.07	0.05	0.18
High	0.03	0.05	0.55	0.009	0.05	0.84
Smoking						
Never-smoker	Ref			Ref		
Past-smoker	0.09	0.04	0.02*	0.04	0.04	0.30
Smoker	0.01	0.05	0.79	0.11	0.04	0.01*
Alcohol						
No	Ref			Ref		
< 1 drink / day	-0.01	0.04	0.69	-0.06	0.04	0.09*
> 1 drink/day	0.2	0.14	0.16	0.08	0.11	0.44
Newborn's gender						
Male	Ref			Ref		
Female	0.05	0.03	0.15	-0.001	0.03	0.97
Season of delivery						
Fall	Ref			Ref		
Winter	-0.013	0.05	0.79	0.02	0.04	0.55
Spring	-0.09	0.04	0.04*	0.007	0.05	0.87
Summer	-0.09	0.04	0.04*	0.007	0.04	0.85

β-estimate is an absolute change in percentage of levels of 8-OHdG or mtDNA content

*Associations statistically significant with *p*-values lower than 0.01.

3.3 Mitochondrial 8-OHdG in maternal blood in association with PM_{10} exposure

In unadjusted analysis we observed a significant positive association of mitochondrial 8-OHdG with PM_{10} exposure during the entire pregnancy (Figure 5A). Each IQR increase in PM_{10} exposure was associated with an increase in mitochondrial 8-OHdG levels of 18.8% (95% confidence interval (CI): 6.5, 32.5, p = 0.002)Table 5 Estimated change of mitochondrial 8-OHdG levels in maternal blood in association with PM_{10} exposure during pregnancy.(Table 5) PM_{10} exposure for the separate trimesters of pregnancy were not significantly associated (p > 0.11) with maternal mitochondrial 8-OHdG levels, but we observed a positive trend in trimester 3 (Figure 5B).

In the adjusted model (Table 5), in which we correct for maternal age, gestational age, maternal education, smoking status, alcohol consumption and season of delivery, maternal mitochondrial 8-OHdG remained positively associated with PM_{10} exposure during the entire pregnancy. Each IQR increase in PM_{10} was associated with an increase maternal 8-OHdG level of 23% (95% CI: 8.4, 39.6 p = 0.002).

In addition, an increase in 8-OHdG levels in maternal blood was positively associated with PM_{10} exposure during the third trimester of pregnancy (34.5%, 95% CI: 10.", 56.7 p = 0.01, for each IQR increment of PM_{10} exposure).

Exposure during the first and second trimester of pregnancy were not significantly (p > 0.09) associated with maternal mtDNA 8-OHdG levels, although a positive trend is shown.

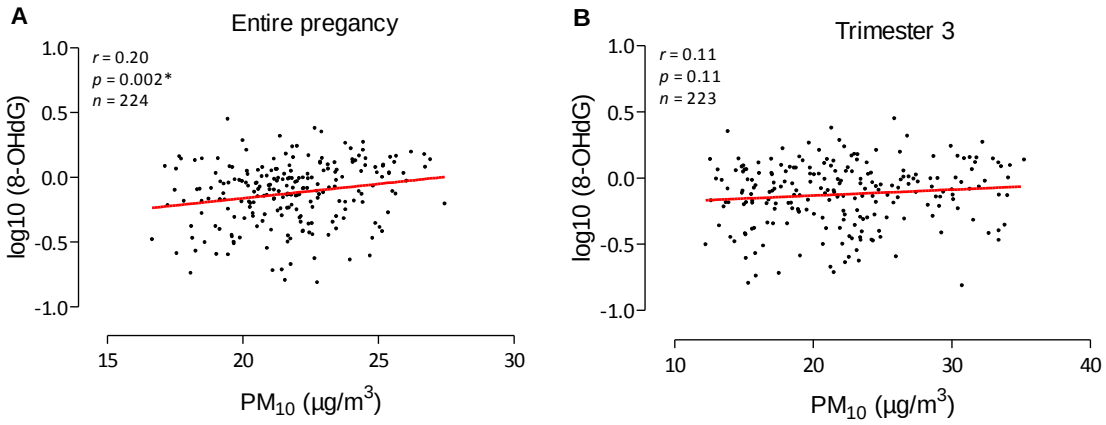

Figure 5 Association between mitochondrial 8-OHdG in maternal blood and PM_{10} exposure(µg/m^3) PM_{10} exposures are presented with Pearson correlation coefficients for the entire pregnancy pregnancy (**A**) and last trimester of pregnancy (**B**). 8-OHdG levels are assessed by the difference between C_T-values of treated and non-treated samples. C_T-values are corrected for inter-run variability and delta C_Ts are $\log_{10}$ transformed. *Correlations are supposed to be statistically significant with p-values lower than 0.05

3.4 Mitochondrial DNA content in maternal blood in association with in PM_{10} exposure

In unadjusted model, we observed a positive correlation between mtDNA content in maternal blood and levels of PM_{10} exposure during the third trimester and last month of pregnancy (Figure 6B) Each IQR increment of maternal PM_{10} exposure during the third trimester and last month of pregnancy was significantly associated with an increase in mtDNA content levels of respectively 14% (95% CI: 3.3, 25.8, p = 0.01) and 11.3% (95% CI: 1.2, 22.5, p = 0.03). PM_{10} exposures during the first and second trimester of pregnancy were not significantly associated (p > 0.15) with maternal mtDNA content levels but we observe an positive trend among the entire pregnancy.(

Table 6). Each IQR increment of PM_{10} exposure during the third trimester and last month was associated with an increase of maternal mtDNA content of respectively 29.6% (95% CI: 10.9, 51.4, p = 0.001) and 16.8% (95% CI): 3.7, 31.5, p = 0.01).

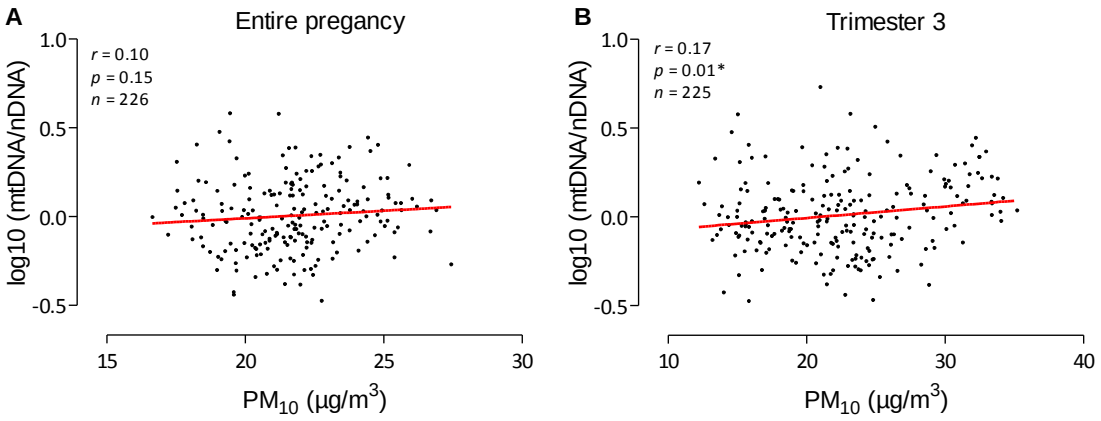

Figure 6 Association between mtDNA content in maternal blood and PM_{10} exposure(µg/m^3) PM_{10} exposures are presented with Pearson correlation coefficients for the entire pregnancy pregnancy (**A**) and last trimester (**B**). Values of mtDNA content are normalized by nDNA content, corrected for inter-run variability and log_{10} transformed. *Correlations are supposed to be statistically significant with p-values lower than 0.05.

Table 5 Estimated change of mitochondrial 8-OHdG levels in maternal blood in association with PM_{10} exposure during pregnancy.

Time window PM_{10}	Unadjusted model			Adjusted model[b]		
	Percent change [a]	**95% CI**	***p*-Value**	**Percent change** [a]	**95% CI**	***p*-Value**
Trimester 1 (1-13w)	4.1	-6.0, 15.3	0.44	8.5	-7.1, 26.7	0.31
Trimester 2 (14-26w)	9.4	-2.1, 22.2	0.11	15.4	-2.2, 36.2	0.09
Trimester 3 (27w-delivery)	9.5	-2.0, 22.5	0.11	31.4	10.3,56.7,	0.003*
Last month	1.6	-8.9, 13.3	0.78	9.1	-5.2, 25.5	0.22
Entire pregnancy	18.8	6.5, 32.5	0.001*	23.0	8.4, 39.6	0.002*

[a] The effect size is calculated as a relative percent change for each IQR increase of PM_{10} exposure ($\mu g/m^3$) at mother's residence during the different time windows.

[b] Adjusted for covariates including maternal age, gestational age, smoking status, maternal education, alcohol consumption during pregnancy and season of delivery.

*Statistically significant with p-value < 0.05.

Table 6 Estimated change of mtDNA content in maternal blood in association with PM_{10} exposure during pregnancy.

Time window PM_{10}	Unadjusted model			Adjusted model[b]		
	Percent change [a]	**95% CI**	***p*-Value**	**Percent change** [a]	**95% CI**	***p*-Value**
Trimester 1 (1-13w)	-2.6	-10.9, 6.6	0.57	-4.3	-16.7, 9.8	0.53
Trimester 2 (14-26w)	-1.7	-11.1, 8.6	0.73	-2.4	-15.7, 13.1	0.75
Trimester 3 (27w-delivery)	14.0	3.3, 25.8	0.01*	29.6	10.9, 51.4	0.001*
Last month	11.3	1.2, 22.5	0.03*	16.8	3.7, 31.5	0.01*
Entire pregnancy	7.6	-2.7, 18.9	0.15	8.0	-3.6, 21.0	0.19

[a] The effect size is calculated as a relative percent change for each IQR increase of PM_{10} exposure ($\mu g/m^3$) at mother's residence during the different time windows.

[b] Adjusted for covariates including maternal age, gestational age, smoking status, maternal education, alcohol consumption during pregnancy and season of delivery.

*Statistically significant with p-value < 0.05.

3.5 Association between mitochondrial DNA content and mitochondrial 8-OHdG in maternal blood

To explore the functional significance of the association between mitochondrial 8-OHdG with exposure to PM_{10}, we evaluated the association between mtDNA content in maternal blood, a measure of damaged dysfunctional mitochondria, and 8-OHdG. Pregnant mothers with high levels of 8-OHdG exhibited high levels of mtDNA content in their blood Figure 7shows a strong association between levels of mtDNA content and 8-OHdG (r=0.48, p = <0.0001). After adjustment of the aforementioned variables, the association remained statistically significant (β =0.54 ± 0.05, p < 0.001).

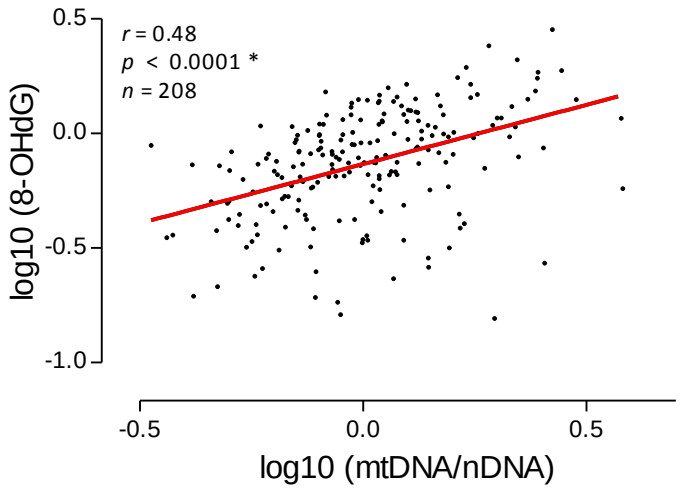

Figure 7 Association between mitochondrial 8-OHdG and mtDNA content in maternal blood. 8-OHdG levels were determined by the difference between C_T-values of treated and non-treated samples. C_T-values are corrected for inter-run variability and delta C_Ts are log_{10} transformed, mtDNA content is normalized by nDNA content, corrected for inter-run variability and log_{10} transformed. *Association supposed to be statistically significant with p-values lower than 0.05.

3.6 Mediating effects of mitochondrial 8-OHdG on the association of PM_{10} and mitochondrial DNA content in maternal blood

A mediation analysis was performed to formally test whether the associations of PM_{10} exposure and mtDNA content were mediated by 8-OHdG in mitochondrial DNA. In mediation analysis, 8-OHdG was estimated to mediate 42% of the positive association between PM_{10} exposure during the third trimester of pregnancy and higher mtDNA content (Figure 8C) No significant mediation has been observed during first and second trimester of pregnancy. During the entire pregnancy, 98% of the association between PM_{10} exposure and a higher mtDNA content was mediated by 8-OHdG (Figure 8D,).

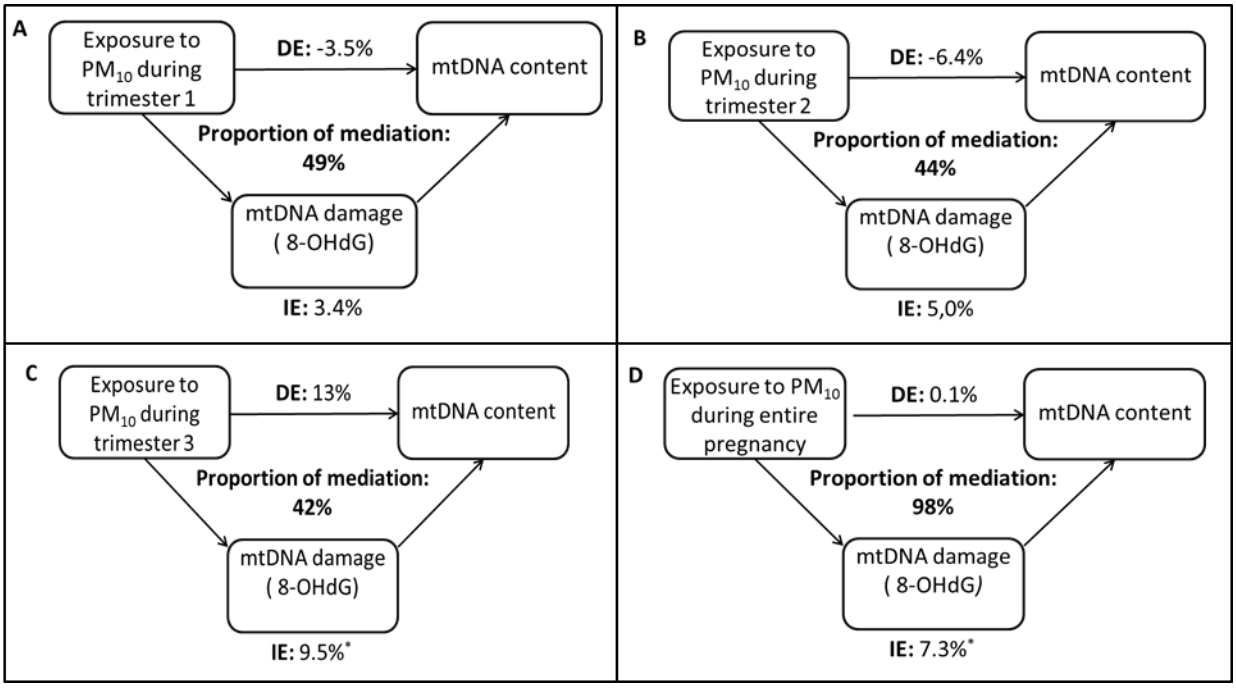

Figure 8 Mediation of the effect of the PM_{10} exposure on mtDNA content through changes in 8-OHdG. The figure displays mitochondrial 8-OHdG as mediator, the estimates of indirect effect (IE), the estimates of the direct effect (DE) and proportion of mediation (IE/DE+IE). The effects are relative changes in maternal mtDNA content for an increment between the IQR of PM10 exposure. The mediation model was adjusted for maternal age, gestational age, smoking status, maternal education, alcohol consumption during pregnancy and season of delivery. *Correlations are supposed to be statistically significant when *p*-values are lower than 0.05.

3.7 Translating effects to the newborn: Association of mitochondrial 8-OHdG with PM exposure and mitochondrial DNA content in cord blood

To investigate the effect we observed in maternal blood are passé trough or translated to the foetus, we determined levels of 8-OHdG and mtDNA content in cord blood of 295 newborns. The adjusted model included the aforementioned variables plus newborns gender an delivery date showed an association between levels of 8-OHdG in cord blood and PM_{10} exposure. For the first and second trimester, a significant positive association of 21.%(95% CI: 5.4, 40.7, p = 0.01) and 18.5% (95% CI: 0.9, 39.0, p = 0.04) respectively has been found. Moreover, during the entire pregnancy, we a show positive trend of increased levels of 8-OHdG in cord blood with PM_{10} exposure. (Table 7) Secondly we observed a significant positive association of mitochondrial 8-OHdG with mtDNA content in cord blood (r =0.58; $p < 0.001$) (Figure 9). The association remained statistically significant after adjustment of the aforementioned variables (β = 0.62±0.05, $p < 0.0001$). However, we did not observe an association between mtDNA content in cord blood and PM exposure during each trimester ($p \geq 0.16$). Therefore, we did not perform a mediation analysis.

Lately placental tissue, we did not observe an association between mitochondrial 8-OHdG and either placental mtDNA content or PM_{10} exposure (r= 0.22 p ≥ 0.06 and r= 0.14 p ≥0.40 respectively).

Table 7 Estimated change in of mitochondrial 8-OHdG levels of in cord blood in DNA in association with PM10 exposure during pregnancy

	Unadjusted model			Adjusted model[b]		
Time window PM_{10}	**Percent change** [a]	**95% CI**	***p*-Value**	**Percent change** [a]	**95% CI**	***p*-Value**
Trimester 1(1-13w)	12.6	3.3,22.8	0.01	21.8	5.4,40.7	0.01*
Trimester 2 (14-26w)	-10.3	-20.1,0.6	0.07	18.5	0.9,39.3	0.04*
Trimester 3 (27w-delivery)	-14.6	-22.9,-5.4	0.003	-14.6	-27.4,0.5	0.06
Last month	-2.8	-11.6, 6.9	0.57	-3.4	-13.4,7.8	0.54
Entire pregnancy	-8.1	-17.3, 2.0	0.11	9.55	-3.1,23.8	0.15

[a] The effect size is calculated as a relative percent change for each IQR increase of PM_{10} exposure ($\mu g/m^3$) at mother's residence during the different time windows.

[b] Adjusted for covariates including maternal age, gestational age, smoking status , maternal education, alcohol consumption during pregnancy, season of delivery, date of delivery and newborn gender.

*Statistically significant with *p*-value < 0.05.

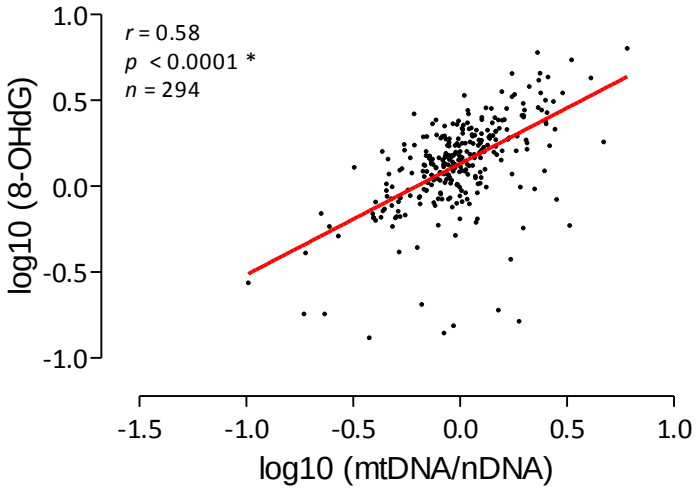

Figure 9 Association between mitochondrial 8-OHdG and mtDNA content in umbilical cord blood.8-OHdG levels are assessed by the difference between C_T-values of treated and non-treated samples. C_T-values are corrected for inter-run variability and delta C_Ts are log_{10} transformed. mtDNA content is normalized by nDNA content, corrected for inter-run variability and log_{10} transformed. *Statistically significant with p-value < 0.05.

3.8 Sensitivity analysis

We applied all our statistical models again replacing PM_{10} exposure(resolution10X10m grids) by $PM_{2.5}$ exposure (4X4km grids) and observed the same results in every exposure window. Supplementary table S3 shows an association between levels of 8-OHdG and PM_{10} exposure during third trimester of pregnancy and entire pregnancy. Supplementary S4 shows the association between mtDNA content and PM_{10} exposure during the last trimester of pregnancy.

4 Discussion

A strong body of evidence associates ambient PM exposure with a wide range of negative health effects in adulthood and also adverse foetal outcomes. The impact of PM pollution on pregnant women itself have been poorly investigated. In this study we investigate both maternal and foetal matrices which could help to understand the effects of *in utero* PM exposure both the health of the mother and unborn child. The present study is based on a population of 224 pregnant women that were enrolled in the ENVIR*ON*AGE birth cohort. The key finding of our study is that mitochondrial 8-OHdG mediates the association between maternal PM_{10} exposure and increased mtDNA content in maternal blood. Secondly, we observed a positive association between 8-OHdG levels with mtDNA content in cord blood. 8-OHdG levels in cord blood were also positively associated with levels of PM exposure during the first and second trimester of pregnancy.

Our results indicate an intermediate role of 8-OHdG in the pathway linking ambient PM exposure to a changes in mtDNA content in maternal blood. Up to know, we cannot ascribe an intermediate role of 8-OHdG on the association of PM exposure with mtDNA content in either cord blood or placental tissue.

4.1 8-OHdG of mitochondrial DNA in association with particulate matter exposure

In the present study, we observed a positive association between mitochondrial 8-OHdG and PM exposure in maternal blood during the entire pregnancy. Several other studies are consistent with our findings and indicate 8-OHdG as a marker for oxidative DNA damage (Supplementary Table S1). Among these studies exposures in a personal, occupational and environmental setting have been considered. Consistent with our results, most of these studies indicated a positive relation between levels of PM exposure or smoke and urinary levels of 8-OHdG [51-61]. Also in lymphocytes an increase in 8-OHdG has been associated with PM exposure[40, 59]. However, few studies indicated no association between PM exposure and levels of 8-OHdG in urine [58, 59, 62, 63] and blood [23]. These contradictive findings can be clarified by due to the inconsistency in study designs, small sample sizes, and differences in exposure assessment techniques. Therefore, more studies with consistent methodology with representative populations including women and other non-occupationally exposed groups are recommended.

Contrary to our project most studies analysed excretions levels of 8-OHdG in urine instead of 8-OHdG levels in mtDNA. Nevertheless, the results of the majority of studies performed with urine are in line with our results of 8-OHdG in mitochondria. This project indicates the association of PM exposure during the entire pregnancy and levels of 8-OHdG in mDNA. To the best of our knowledge this is the first study which was able to associate PM exposure to 8-OHdG in mtDNA. The advance of measuring 8-OHdG in mtDNA compared to the excreted form of 8-OHdG is that by the DNA repair mechanism in mtDNA is less efficient contrary to the one of nDNA[34]. Consequently, the 8-OHdG lesions in mtDNA are more persistent and able to accumulate among a long period of time which could explain the association with PM levels related to the entire pregnancy rather than trimester specific associations[64].

4.2 Mitochondrial DNA content in association with particulate matter exposure

The association between PM exposure and changes in mtDNA content have already been investigated by several studies as demonstrated in Supplementary Table S2. Hou *et al*(2010) found a positive association between environmental PM exposure and mtDNA[65] n contrast a decrease in mtDNA content with PM levels in the blood of highly exposed individuals [66], in placental tissue during in the last period of pregnancy [67] and to PAH, an anorgranic fraction of PM, in white blood cells [68].Occupational exposure studies showed a significant increase of mtDNA content in blood cells in association with PM exposure[65] and benzene exposure [69]. Smoking is a direct source of personal exposure to PM. Masayesva *et al.* (2006) showed that smoking increased the mtDNA content in saliva cells [70] Moreover an increase in mtDNA content in the cells of lung tissue cells of light smokers has been shown, but a decrease in the lung tissue of heavy smokers[71].

Taken together, our findings demonstrated association between PM and mtDNA content and re consistent to other research. Contradicting results of an increase or decrease mtDNA content can be explained by differences in exposure levels and the investigated matrix.

As mentioned before, oxidative stress can be induced by PM. An increase of mtDNA content is suggested as a response to the increased amount of ROS in order to compensate for damage and to encounter the increased respiratory demand which is required for ROS clearance [25, 34].The trier model (Figure 3) explains that once ROS concentrations rise up beyond the threshold, defence mechanisms of the cell are is not sufficient anymore to remove these high concentrations of ROS and lead to cytotoxicity and apoptosis which could be reflected in a decrease in mtDNA content. This interpretation is visualized in Figure 10. Moreover tissue, with lower cell turnover compared to other matrixes could point to a lower ROS clearance and consequently explain the lower levels of mtDNA content [72].

4.3 Windows of susceptibility

In our study, mtDNA content was associated to maternal PM_{10} exposure levels during the last trimester of pregnancy which might suggest a potential window of susceptibility of PM_{10} exposure during pregnancy. Moreover, this finding might point to a rather short- term effect of PM_{10} on mtDNA content levels in maternal blood.

In contrast the effect of PM_{10} on 8-OHdG in mtDNA is supposed to be more resistant due to the impaired DNA repair in mitochondria [64]. Our findings show an increasing trend in the relation between PM_{10} exposure and 8-OHdG level during the entire pregnancy, which could point to an accumulation of this long-term biomarker for oxidative mtDNA damage. These data indicate certain windows of susceptibility in which the individual is more prone to damaging effects op PM exposure.

4.4 Mediating effects of 8-OHdG on the association of PM_{10} and mitochondrial DNA content

To our best knowledge, this is the second study showing an association between levels of mtDNA content and 8-OHdG. Lin et al.(2008)[43] indicated a significant correlation between mtDNA content and the levels of 8-OHdG in lungs cancer tissues of 29 patients. Our mediation analysis suggests that mitochondrial 8-OHdG is an intermediate factor of changes in mitochondrial DNA content. Consequently the formation of oxidative mtDNA damage, exemplified by 8-OHdG, might be an important mediator linking PM-exposure to levels of mtDNA content. Moreover, these results confirm the suggested importance of oxidative stress as driver of the negative effects of PM pollution (Figure 10).Figure 10

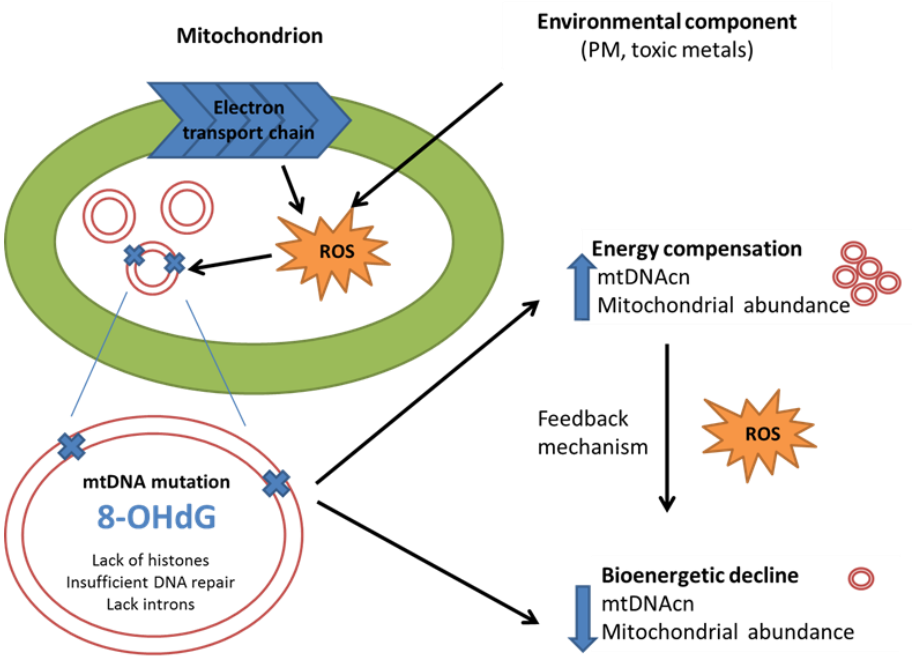

Figure 10 Suggested intermediate mechanism of altered mtDNA content due to PM exposure. In addition to ROS derived from the election transport chain, the production of ROS can be increased by environmental factors including PM. ROS will react with the highly vulnerable mtDNA and induce 8-OHdG lesions, the most common form of oxidative DNA mutation. Mutation in the mtDNA will result in mitochondrial stress and dysfunction. In order to compensate for this impairment and to encounter the higher energy demands the mitochondrial DNA copy number and mitochondrial abundance will increase. This can cause an extra increase in the levels of ROS and once beyond a certain threshold, the compensating response will not be able anymore to reduce the damaging effects of ROS. This eventually will lead to cytotoxicity and apoptosis and consequently a decrease in mtDNA content.

4.5 Relevance of particulate matter-induced effects to the newborn

Besides the suggested vulnerability of the mother during pregnancy for the negative heath effect of PM, the health of the foetus is of great concern considering the systemic inflammation and possible transplacental transport of PM [73]. Moreover a growing body of evidence associates prenatal PM exposure to adverse foetal effects including low birth weight (LBW), intrauterine growth retardation (IUGR) [22]. These birth outcomes are relevant indicators the health status of individuals in adult life, including elevated mortality and morbidity in childhood and an increased risk of hypertension, coronary heart disease, and non-insulin-dependent diabetes in adult life.[45]

We showed a positive association between levels of 8-OHdG and mtDNA content in cord blood. Furthermore, we observed a significant effect of PM exposure during the first two trimesters of pregnancy and elevated 8-OHdG levels again which can point to long persistence of this marker. We did not found an association between PM_{10} exposure and mtDNA content and therefore we were did not perform a mediation analysis. Moreover, none of these associations have been found of in placental tissue which suggest another intermediate mechanism.

Several transplacental mechanisms are suggested to be induced in mothers exposed to air pollutants and underlie adverse birth outcomes, including formation of DNA adducts, displacement of the oxygen–hemoglobin dissociation curve, inflammation and direct toxic effects on fetuses and the placenta [73]. Placentas of mothers living in a highly air polluted district, showed higher DNA adduct levels compared to mothers form a less polluted area. High levels of DNA adducts were associated with reduced gestational length, and a correlation between the adduct levels in the mother's and the newborn's blood(umbilical cord blood) has been observed [20, 73]

4.6 Future perspectives

Interestingly, this is the first study describing a possible intermediate mechanism of altered mtDNA content and PM_{10} exposure. However, we cannot ascertain this link and other studies need to be conducted to confirm these findings. In order to further investigate and establish the effects of PM exposure on mitochondria, experimental studies using exposed cell lines can be of great interest. Moreover, to investigate the maternal vulnerability and temporal changes in mtDNA content and 8-OHdG with exposure to PM, the collection of maternal blood samples on different time points during the pregnancy would be of great interest. In this way further understanding can be obtained among the fluctuations of mtDNA content and 8-OHdG during the entire pregnancy and the impact of oxidative mtDNA damage due to maternal PM exposure.

4.7 Strengths and limitations

Limitation of the project concerns the recruitment on of the mother just at the end of their pregnancy which consequently impairs the ability to investigate changes in the mtDNA content and 8-OHdG in mitochondria DNA among the entire pregnancy.

In contrast to blood leukocytes, platelets do not contain a nucleus and therefore platelet contamination increases mtDNA without contributing to nDNA and distort the ratio of mtDNA content to nDNA content.

The strength of this study is the large amount participants and collected information from these subjects. In this way we were able to set up relevant statistical model in which we could correct for several variables that could influence the outcomes.

Secondly the mediation analysis we used in this study is a validated statistical models an gives us the opportunity to investigate intermediate mechanisms of an exposure-outcome relationship[50].

5 Conclusion

PM pollution is strongly associated with several negative health effects in adults and also adverse foetal outcomes. Contrary to previous studies this study focused on the maternal response to PM exposure during pregnancy before focussing on effects on the foetus. As a result we provide new insights into the mechanisms of altered mitochondrial function in response to PM exposure. Mediation analysis confirmed mtDNA damage as an intermediate mechanism for PM_{10} induced mtDNA alterations in maternal blood. Furthermore we also suggest an important role of mtDNA damage in cord blood, exemplified by increased levels of 8-OHdG. However further research should be performed to better understand the underlying mechanisms and the translation of the maternal effects of PM to the foetus.

6 References

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7 Supplemental information

Table S5 Relevant studies concerning the association between PM and mitochondrial 8-OHdG.

	Exposure	8-OHdG	Matrix	Subjects	Reference
Personal exposure	Smoking	↑	Urine	182 healthy smokers	Priemé H. *et al*.1998 [51]
				83 randomly selected healthy subjects)	Loft S. *et al.* 1992[52]
				1076 participants	Kanaya S. *et al*. 2004 [53]
				smokers and non-smokers	Yao Q. *et al* 2004 .[54]
Occupational exposure	Exposed vs. controls	↑	Urine	47 female highway toll workers, 27 female office workers	Lai *et al*. 2004[55])
				57 non-smoking diesel bus drivers in urban vs. rural areas	Loft *et al*. 1999 [56]
	PM	↑	Urine	20 boilermakers	Kim *et al*. 2004[57]
	PM,PAH, EC	-	Urine	32 bus maintenance workers	Sauvain *et al*. 2011[58]
Environmental exposure	PM	-	Urine	49 non-smoking students	Sorensen *et al*. 2003 [59]
				15 compromised subjects 6 healthy subjects	Kim *et al*. 2009[63]
	PM	↑	Urine	894 children	Svecova *et al*. 2009[74]
				320 military veterans	Ren *et al*. 2011[60]
				125 students	Huang *et al*. 2012[61]
	PM	↑	Lymphocytes	49 non-smoking students	Sorensen *et al*. 2003[59]
				51 healthy nonsmokers	Vattanasit *et al*. 2013 [40]
	PM	-	Blood	76 young healthy students	Chuang *et al*. 2007[23]
	PAH	↑	Urine	894 children	Svecova *et al*. 2009[75]
	PAH	↑	Lymphocytes	51 healthy nonsmokers	Vattanasit *et al*. 2013[40]

Table S6 Relevant studies concerning the association between PM an mtDNA content.

	Exposure	mtDNA content	Matrix	Subjects	Reference
Personal exposure	Smoking	↑	Exfoliated cells in saliva	604 subjects with varied smoking exposure	Masayesva BG *et al*. 2005[70]
	Light smoking	↑	Lung tissue cells	Light smokers	Lee HC *et al*. 1998[71]
	Heavy smoking	↓	Lung tissue cells	Heavy smokers	
	Maternal smoking	↓	Placental tissue cells	9 smoking and 19 nonsmoking mothers	Bouhours-Nouet N *et al*. 2005[76]
Occupational exposure	PM	↓	Blood cells	60 truck drivers and 60 office workers	Hou *et al*. 2013[66]
	Low benzene	↑	Blood cells	341 individuals from occupational groups with low-level benzene exposures and 178 referents	Carugno *et al*. 2012[69]
Environmental exposure	PM	↑	Blood cells	63 male healthy steel workers	Hou *et al*. 2010[65])
	PAH is house dust	↓	White blood cells	46 persons	Pieters N. *et al*. 2013[68]
	PM	↓	Placental tissue cells	178 mother –newborn pairs	Janssen *et al*.2012 [67]
	PM	-	White blood cells in cord blood	178 mother –newborn pairs	

Table S7 Estimated change of mitochondrial 8-OHdG levels in maternal blood in association with $PM_{2.5}$ exposure during pregnancy

	Unadjusted model			Adjusted model[b]		
Time window $PM_{2,5}$	**Percent change** [a]	**95% CI**	**p-Value**	**Percent change** [a]	**95% CI**	**p-Value**
Trimester 1 (1-13w)	3.8	-4.2, 12.5	0.36	6.9	-5.1, 20.4	0.27
Trimester 2 (14-26w)	8.4	-1.2, 18.8	0.09	13.0	-1.3, 29.3	0.08
Trimester 3 (27w-delivery)	8.7	-1.7, 20.3	0.11	24.0	6.5, 44.4	0.01*
Last month	3.8	-6.4, 15.1	0.48	11.2	-2.7, 27.1	0.12
Entire pregnancy	8.2	1.4, 15.3	0.02*	8.92	1.0, 17.4	0.03*

[a] Effect size was estimated for each Interquartile increase (IQR, Q3-1)increase in $PM_{2,5}$ exposure at mother's residence during the different indicated periods of pregnancy

[b] Model adjusted for covariates including maternal age, smoking status , maternal education, alcohol consumption during pregnancy, season of delivery and gender of the new-born.

* Statistically significant with *p*-value < 0.05

Table S8 Estimated change of mtDNA content in maternal blood in association with $PM_{2.5}$ exposure during pregnancy:

	Unadjusted model			Adjusted model[b]		
Time window $PM_{2,5}$[a]	**Percent change** [a]	**95% CI**	**p-Value**	**Percent change** [a]	**95% CI**	**p-Value**
Trimester 1 (1-13w)	-0.4	-9.1, 9.1	0.93	0.4	-12.1, 14.7	0.95
Trimester 2 (14-26w)	-0.1	-1.1, 1.0	0.90	-3.5	-17.8, 13.3	0.66
Trimester 3 (27w-delivery)	1.4	0.4, 2.4	0.01*	27.9	9.9, 48.8	0.002*
Last month	1	0.2, 1.8	0.02*	15.0	4.3, 26.8	0.01*
Entire pregnancy	1.2	-0.5, 2.9	0.18	3.6	-3.5, 11.3	0.33

[a] Effect size was estimated for each Interquartile increase (IQR, Q3-1)increase in $PM_{2,5}$ exposure at mother's residence during the different indicated periods of pregnancy

[b] Model adjusted for covariates including maternal age, smoking status , maternal education, alcohol consumption during pregnancy, season of delivery and gender of the new-born.

* Statistically significant with *p*-value < 0.05

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Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2014**

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Grevendonk, Lotte

Datum: **10/06/2014**