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GENEESKUNDE

master in de biomedische wetenschappen: milieu en gezondheid

Masterproef

Decreased placental mitochondrial DNA-content in response to air pollution during in utero life

Promotor :
Prof. dr. Tim NAWROT

Copromotor :
dr. Karen SMEETS

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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

Maastricht University

universiteit hasselt
UNIVERSITEIT VAN DE TOEKOMST

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Content

Abbreviation list

36B4	Acidic ribosomal phosphoprotein P0
β-act	β-actin
AC	Abdominal contour
As	Arsenic
ATPase	F_1F_0-ATP synthase
BPD	Biparietal diameter
Cd	Cadmium
COX	Cytochrome c oxidase
Cr	Chromium
C_T	Cycle Threshold
CYP1A1	Cytochrome P450 A1 enzyme
Cyt b	Cytochrome b
D-loop	Displacement-loop
FL	Femur length
GADPH	Glyceraldehyde-3-phosphate
H_2O_2	Hydrogen peroxide
HBG-1	Haemoglobin gamma A
HC	Head circumference
HO°	Hydroxyl radical
IARC	International Agency for Research on Cancer
IRC	Inter-run calibrator
KMI	Koninklijk Meteorologisch Instituut
mtDNA	Mitochondrial DNA
mtDNAcn	Mitochondrial DNA copy number
ND	NADH dehydrogenase
nDNA	Nuclear DNA
nDNAcn	Nuclear DNA copy number
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa B
NH_3	Ammonia
Ni	Nickel
NO_x	Nitrogen oxides
NTC	Non template control
$O_2°^-$	Superoxide anion
OFD	Occipital-frontal diameter
PAH	Polycyclic aromatic hydrocarbons
Pb	Lead
PM	Particulate matter
PM_{10}	Particles with a diameter smaller than 10 µm
$PM_{2.5}$	Particles with a diameter smaller than 2.5 µm
$PM_{0.1}$	Particles with a diameter smaller than 0.1 µm
PPARγ	Peroxisome proliferator-activated receptor gamma
PGC-1α	PPAR coactivator alpha
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SO_x	Sulphide oxides
TCD	Transcerebral diameter
UFP	Ultrafine particle
ZOL	Ziekenhuis Oost-Limburg

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Acknowledgments

This thesis project would not have been possible without the support of many people. In this section, I would like to thank each and everyone that made this opportunity for me to come true.

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Summary

INTRODUCTION: Ambient air pollution consists of gaseous and particle pollutants, originating from natural and anthropogenic (industry, traffic) sources. Particulate matter (PM) consists of many toxic constituents and is most relevant to human health. PM_{10} exposure contributes to respiratory and cardiovascular morbidity. Acute- and long-term exposure to PM has also been linked to increased hospitalization and mortality from susceptible people. Numerous studies have emphasized the importance of PM, and its associated metal components, in the formation of reactive oxygen species (ROS) that influence biological processes. Mitochondria are the major intracellular sources and primary targets of ROS. Mitochondrial DNA (mtDNA) is particularly vulnerable to ROS-induced damage and has a high mutation rate. Additionally, mtDNA-content is very important for maintaining normal mitochondrial function and meeting the energy needs of cells. Given its multiple essential roles in metabolic pathways, mitochondrial dysfunction has been linked to the development of various diseases including type II diabetes, breast cancer, multiple sclerosis and adverse foetal outcomes.

OBJECTIVES: In this cross-sectional study, we investigated the influence of PM_{10} exposure during pregnancy on the mtDNA-content, an established marker of mitochondrial damage and dysfunction. We hypothesized that mtDNA-content changed during pregnancy in response to PM_{10} exposure and may underlie susceptibility to mitochondrial dysfunction.

METHODS: DNA was extracted from placental tissue and leukocytes of umbilical cord blood from 178 mother-newborn pairs and was quantified with real-time PCR. mtDNA-content was determined by amplification of two mitochondrial genes (MTF3212/R3319 and ND-1) and adjusted for three single-copy nuclear control genes (36B4, β-act and HBG-1).

RESULTS: We found a significant association between placental mtDNA-content and exposure to PM_{10} during the last period of pregnancy. Each 10 $\mu g/cm^3$ increase in PM_{10} during the third trimester of pregnancy was associated with a lower placental mtDNA/nDNA ratio of $\beta = -0.161$ (95 % CI: -0.094 to -0.229; $p < 0.0001$). In contrast, no association between mtDNA-content in cord blood and PM_{10} exposure was found. Furthermore, we found an association between distance to major roads and placental mtDNA-content. Each doubling in distance was associated with an increase in mtDNA/nDNA ratio by 5.8 % (95 % CI: 0.2 % to 6.5 %, $p = 0.0447$).

CONCLUSIONS: To the best of our knowledge, this is the first study reporting the effects of PM_{10} exposure on placental mtDNA-content. Our findings indicate a potential window for susceptibility by trimester exposure that may adversely affect mitochondrial function and possibly foetal outcome. mtDNA-content is associated with distance to major road, a surrogate for traffic-related air pollution.

Samenvatting

INLEIDING: Luchtverontreiniging is een heterogeen mengsel van gas- en deeltjesvormige substanties die gevormd worden door natuurlijke en antropogene bronnen zoals industriële activiteiten en verkeer. Fijne stofdeeltjes (PM) bestaan uit verschillende toxische componenten die schadelijk zijn voor de gezondheid. Blootstelling aan PM_{10} draagt bij tot de ontwikkeling van respiratoire and cardiovasculaire ziekten die kunnen leiden tot verhoogde ziekenhuisopnames en mortaliteit. Recente studies benadrukken het belang van PM in de vorming van reactieve zuurstofsoorten (ROS). Mitochondria zijn de voornaamste bronnen en doelwitten van ROS. Mitochondriaal DNA (mtDNA) is meer vatbaar voor ROS-geïnduceerde schade dan nucleair DNA (nDNA) en leidt tot een verhoogde mutatie snelheid. Bijgevolg hangt het functioneren van de mitochondria af van de mtDNA-inhoud. Mitochondria spelen een essentiële rol in verschillende metabolische pathways. Recente studies tonen een relatie tussen mitochondriale disfunctionering en de ontwikkeling van ziektes zoals type II diabetes, borstkanker, multiple sclerose en laag geboortegewicht.

OBJECTIEVEN: In deze cross-sectionele studie hebben we de invloed van PM_{10} blootstelling tijdens de zwangerschap op de mtDNA-inhoud van placenta weefsel en leukocyten van navelstrengbloed onderzocht. mtDNA-inhoud is een marker van mitochondriale schade en disfunctie.

METHODEN: DNA werd geëxtraheerd uit cellen van 178 moeder-pasgeborene paren en gekwantificeerd met behulp van real-time PCR. mtDNA-inhoud werd bepaald door twee mitochondriale genen (MTF3212/R3319 and ND-1) te amplificeren en te normaliseren met drie nucleaire referentie genen (36B4, β-act and HBG-1).

REULTATEN: Er werd een associatie gevonden tussen de mtDNA-inhoud van placenta weefsel en blootstelling aan PM_{10} gedurende de laatste periode van de zwangerschap. Elke verhoging van PM_{10} met 10 $\mu g/cm^3$ tijdens het derde trimester van de zwangerschap ging gepaard met een lager mtDNA/nDNA ratio van $\beta = -0.161$ (95 % CI: -0.094 to -0.229; $p < 0.0001$). Er werd geen relatie gevonden tussen mtDNA-inhoud van navelstrengbloed en PM_{10} blootstelling. Verder werd er een associatie gevonden tussen afstand tot grote wegen en mtDNA-inhoud van placenta weefsel. Elke verdubbeling in afstand ging gepaard met een verhoging van de mtDNA/nDNA ratio met 5.8 % (95 % CI: 0.2 % to 6.5 %, $p = 0.0447$).

CONCLUSIES: Dit is de eerste studie die in een epidemiologische context een associatie aantoont tussen PM_{10} blootstelling en mtDNA-inhoud van placenta weefsel. Onze bevindingen wijzen op een potentieel kritische periode voor blootstelling aan PM_{10} tijdens de zwangerschap, dat gevolgen kan hebben op de mitochondriale functie en foetale gezondheid. mtDNA-inhoud is geassocieerd met afstand tot drukke wegen, een surrogaat voor verkeers-gerelateerde luchtverontreiniging.

1 Introduction

Industrial activities and mining increased since the beginning of the industrial revolution in the late 19th and early 20th century[1]. Together with this fast growing industry, the problem of traffic-related air pollution raised extensively. Although ambient air quality has improved considerably during the last decades in most European countries[2], the effects of airborne particulate matter remain to be of greatest concern in terms of human health[1,3-9].

1.1 Particulate matter as a part of total air pollution

The atmosphere is an essential system to support life on Earth. All air contained in the atmosphere is a complex, dynamic, gaseous system. Introduction of chemicals, particulate matter or biological materials pollute air and can harm humans or other living organisms.

1.1.1 Classification of air pollutants

Air pollutants originate from natural (geological dust, forest fires, volcanoes, methane) or anthropogenic sources (fossil fuel burning, refineries/power plants, industry, transport). These pollutants consists of both gaseous and particle pollutants[3,10]. Furthermore, a subdivision can be made in the production process of pollutants. Primary pollutants are emitted directly from a process e.g. ash from volcano eruptions, volatile organic compounds, nitrogen oxides (NO_x), sulphide oxides (SO_x) from factories and toxic metals in a metal producing factory. Secondary pollutants are formed due to chemical reactions of UV-light, ozone or from primary gaseous pollutants including SO_2, NO_x, ammonia (NH_3) or organic compounds[5]. These products are less volatile and can form new particles (nucleation) or attach to already existing particles (coagulation)[11,12].

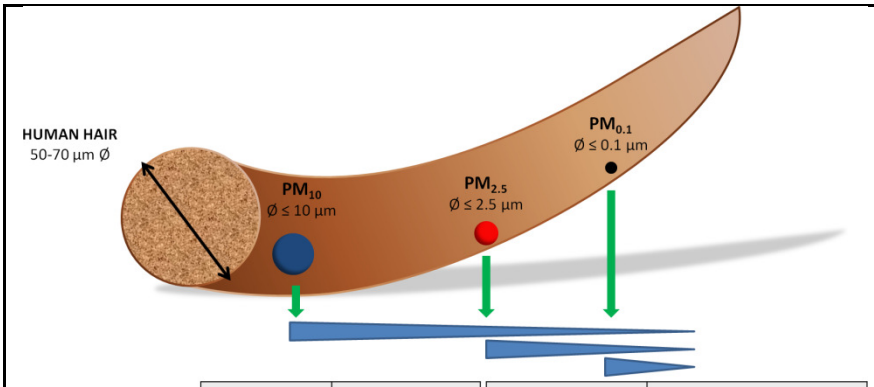
1.1.2 Particulate matter

Particulate matter (PM) is a heterogenic mixture of particles suspended in a gas or liquid droplets that may vary in mass, size, and chemical composition. The characteristics depend on the source, in/outdoor exposure and meteorological conditions (weather, season, etc.)[6]. Relevant to human health, inhalable particles are defined to contain particles with an aerodynamic diameter equal or less than 10 µm and are classified on their size: PM_{10}, $PM_{2.5}$ and $PM_{0.1}$ or ultrafine particles (UFP's).[11,12] (Figure 1)

a) PM_{10} are all particles with an aerodynamic diameter smaller than 10 µm and consist of fugitive/geological dust from roads and industries, biologic material (sea salt, endotoxines, pollen, spores) but encloses also smaller components (diesel exhaust), outlined in the next fractions.

b) $PM_{2.5}$ is defined to include particles with an aerodynamic diameter smaller than 2.5 µm and arise mainly from combustion of fossil fuels used in power generation, industry and diesel engines. $PM_{2.5}$ contain toxic metals, element carbon and reaction products of inorganic compounds (NH_3, NO_2 and SO_2). Moreover, particles are constantly changing and interacting with semi-volatile/volatile compounds such as polycyclic aromatic hydrocarbons (PAHs) that can absorb reactive (transition) metals.

c) $PM_{0.1}$ or UFP's are particles with an aerodynamic diameter smaller than 0.1 µm and are formed during gas-to-particle conversion or during incomplete fuel combustion of diesel engines and contain condensates of toxic metals[13]. Although the composition of UFP's are generally compatible with $PM_{2.5}$, the classification is made due to their small size, large surface area and high alveolar deposition.



Component	Source	Component	Source
Wind-blown dust	Agriculture/Mining/ Unpaved soils	Sulfates	Factories
		Nitrates	Traffic
Sea salt	Sea spray	Ammonium	Agriculture/Traffic
Pollen	Vegetate	Toxic metal	Smelting/Traffic/Combustion/ Metal processing
Spores	Vegetate		
Mold	Fungal	Organic compounds	Combustion
Endotoxins	Bacterial	Element carbon (BC)	Incomplete combustion

Figure 1. A summary of the composition of particulate matter. First, a schematic representation of PM to a human hair based on their size is shown. The number after PM refers to the maximum size in aerodynamic diameter (µm). Second, an overview of the components and source of PM_{10}, $PM_{2.5}$ and $PM_{0.1}$ is given. PAHs and other chemicals in the air are absorbed onto the surface of particles[14]. Black carbon is a primary constituent of diesel PM. Particulate matter (PM); Polycyclic aromatic hydrocarbons (PAHs); Ultrafine particles (UFP's).

The classification of these groups is important to define because these properties govern their source, chemical composition, transport through the air, deposition within the respiratory system and health effects.[2,11,15,16]

Depending on the size, particles can be inhaled and deposited in different parts of the respiratory tract. Particles larger than 10 µm are deposited in proximal airways and eliminated by mucociliairy clearance ending up in the gastrointestinal tract, while PM_{10} and $PM_{2.5}$ are retained in bronchial parts of the lung[10,13,17]. Small $PM_{2.5}$ and UFP's penetrate deeply into pulmonary alveoli by diffusion and increase epithelial permeability, promoting entry of PM into the interstitium and may translocate to the circulation[10,16]. It has been shown that UFP's collected from human lungs consist mainly of metals[18].

1.1.3 Metal components of particulate matter

Essential metals such as copper, iron and zinc are necessary for normal functioning of biomolecules in every living organism whereas toxic metals are non-essential minerals that have no biological role in physiological systems. Excessive exposure to essential and non-essential metals are harmful for organisms[19]

Cadmium

Cadmium (Cd) is a non-essential toxic metal of great concern in the environment because of its toxicity to plants, animals and humans. Emissions date primarily from the past but because of Cd's long half-life, the problem still remains. Cd is emitted in the atmosphere in association with fossil fuel combustion, agricultural application of phosphate fertilizers and as a by-product from refining sulfidic ores of zinc and to a lesser degree of lead (Pb).[20-22] Atmospheric deposition of Cd contaminates soils and water. Cd-polluted soil can be taken up by the wind and generate polluted dust particles. Crops grown on contaminated soils accumulate Cd in their roots and leaves. The major route of human exposure for the non-smoking population occurs through intake of contaminated food products or water where 5 - 8 % is absorbed, but may increase with iron deficiency[23]. Inhalation is the second major route of Cd exposure to humans, 10 - 50 % of the inhaled Cd is absorbed[19,24]. Smokers inhale about 10 % of the Cd content of one cigarette (1 - 2 µg per cigarette) on top of the exposure of non-smokers[24]. The half-life of Cd in the body is 10 - 30 years as a consequence of the storage of metallothionein-bound Cd in the kidneys, liver and testes[23]. Urinary Cd concentration is a biomarker for chronic exposure. The amount of urinary Cd is proportional to the amount of Cd in the kidney whereas Cd levels in the blood reflect the last few months of exposure. Pooled hazard rates of two recent environmental population based cohort studies revealed that for each doubling of urinary Cd concentration, the relative risk for mortality increases with 17 % (95 % CI: 4.2 to 33.1 %; $P < 0.0001$)[25]. Cd-related morbidities are kidney damage[26], osteoporosis resulting from reduced calcium reabsoption in the nephron[26], diabetes[23] and cancer[27].

Chromium, nickel, arsenic and lead

Chromium (Cr), nickel (Ni) arsenic (As) and Pb occur naturally in the environment. Natural atmospheric emissions are due to volcano eruptions, erosions, sea salt and geological dust[22]. However, anthropogenic sources are the major route of Cr, Ni and As emission including metal producing industries, mining, burning of fossil fuels and pesticides (As)[28]. Predominant introduction of Pb into the environment include battery factories, leaded gasoline, old lead pipes and paints[22]. Due to inhalation and ingestion, these metals enter the food chain (except for Ni), causing a widespread distribution in ecosystems. Different oxidation states of the metals govern their chemical

and physiological characteristics. For example, Pb and Cd can interfere with other divalent cations such as calcium^{+II} and disrupt physiological systems. Hexavalent Cr (Cr^{+VI}) and Ni^{+II} are transition metals with strong oxidative properties and exert toxic and mutagenic effects when inhaled. The metals are transported through the blood to different parts of the body and most metals are excreted in the urine. Several health effects are caused by excessive intake and the majority of metals (Cr^{+VI}, Ni, inorganic As and Cd) are classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (group 1). Pb has no essential role in the metabolism but damages the nervous system and causes brain disorders, especially in young children.[19,20,29-31]

1.2 Health effect of particulate matter

Although the gaseous pollutants play a role in the hazardous effects of air pollution, major health problems such as respiratory and cardiovascular conditions are caused by PM. Other less investigated health effects relate to the birth outcome of newborns.

Ambient air quality has improved considerably during the last decades in most European countries[2]. Nevertheless, even at levels commonly achieved nowadays (daily average 50 µg/cm^3, yearly average 20 µg/cm^3), the adverse health effects of traffic-related air pollution still exists[22]. In particular, PM_{10} contributes to respiratory and cardiovascular morbidity[4,5,8,9]. Pulmonary inflammation is a trigger for respiratory diseases including asthma, chronic bronchitis, obstruction of airways and reduced lung function[32,33]. Cardiovascular effects can result in atheromatous plaques, increased viscosity of the blood and thrombosis[34]. Acute- and long-term exposure to PM has also been linked to increased hospitalization, total deaths, cardiopulmonary mortality and lung cancer in susceptible people[6,7]. Predisposed people include elderly, infants and people with pre-existing heart and lung disease. These chronic effects are supported by well-documented epidemiological studies and historical data. One often refers to the Meuse valley fog of 1930 or the London fog of 1952 that led to increased incidence of mortality rates.[34,35]

1.2.1 In utero health effects of particulate matter and the functional role of the placenta

The placenta plays a pivotal role in processes such as growth and organ development and serves as a dynamic protection organ for the foetus. Mitochondria play an important role in the regulation of these processes.[31] Pregnant women are exposed to a variety of foreign chemicals due to medication, smoking, alcohol consumption, occupational and environmental sources. Foetuses are particularly susceptible to toxicants because of their physiologic immaturity and exposure during critical development periods (i.e. higher rates of cell proliferation or changing metabolic capabilities).[36] Chemical compounds can cross the placenta through passive diffusion or transporters between the

maternal and foetal side of the placental membrane[37]. Although the placenta can metabolise some foreign chemicals during pregnancy, others accumulate and affect placental function and morphology[37,38]. Epidemiological studies have shown that exposure to ambient air pollution, mainly PM, affects foetal development[38-42]. Air pollution may affect maternal respiration or general health and, in turn, impair uteroplacental/cord blood flow, nutrient and oxygen transport to the foetus. Foetuses adapt their mitochondrial structure and metabolism when the supply of nutrients is limited. In doing so, this 'metabolic reprogramming' may be at the origin of adverse pregnancy outcomes including low birth weight[43]. Several studies link low birth weight to particulate air pollution[39,41,44,45] and toxic metals[46,47], which are produced by traffic and industrial activities. Barker was the first to put forward the foetal origin hypothesis which states that reduced foetal growth is strongly associated with a number of chronic conditions later in life[48]. Foetal adaptations can result in low birth weight and increase susceptibility to coronary heart disease[49], metabolic syndrome[50], chronic lung disease[51] and osteoporosis[52].

1.3 Molecular pathways of particulate matter

Several mechanisms have been proposed to explain the adverse health effects of PM. These include endotoxin effects, stimulation of capsaicin/irritant receptors, autonomic nervous system activity, pro-coagulant effects and covalent modification of cellular components[4]. Yet, PM-induced DNA damage, as a result of oxidative stress and inflammation, has received the most attention and is believed to lie at origin of various adverse health conditions.

1.3.1 Oxidative stress

The term "oxidative stress" is a disturbance of the cellular redox balance through the inhibition of the anti-oxidative defence system and/or the production of reactive oxygen species (ROS), damaging DNA, proteins, lipids and influencing gene expression. ROS are partially reduced O_2 molecules with high reactivity including the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$)[53]. $O_2^{\bullet -}$ is not particularly damaging but generates H_2O_2 which in turn generates highly reactive oxidizing $HO^{\bullet}$. Peroxynitrite is a reactive nitrogen that is produced by the reaction of nitric oxide with O_2^-. ROS is generated by a large variety of mechanisms, including, ischemia/reperfusion, inflammation, metabolism of foreign compounds and mitochondrial respiration[54]. During the latter, leakage of electrons from the mitochondrial respiratory chain generate highly reactive radicals. Hence, mitochondria are particularly susceptible to ROS-induced damage and affect mitochondrial morphology and mitochondrial DNA replication. Low levels of oxidative stress activate antioxidant defences including superoxide dismutase, glutathione, catalase, etc., whereas higher levels lead to pro-inflammatory and cytotoxic effects[55]. Besides the negative effects, this disturbance in redox

balance plays a role in signalling and transduction pathways such as apoptosis, growth and development, immune responses, inflammation and protection against pathogens[53].

1.3.2 Mechanisms of oxidative stress induced by particulate matter

A number of PM constituents may increase the generation of ROS by a variety of reactions such as (i) transition metal catalyses, (ii) metabolism, (iii) redox-cycling and (iv) inflammation[10,55] (Figure 2).

(i) Soluble transition metals (e.g. Ni, Cr) can reside on the surface of particles and generate ROS through Fenton reactions and act as catalysts by Harber-Weiss reactions:

Net Haber-Weiss reaction: $\bullet O_2 + H_2O_2 \rightarrow \bullet OH + HO^- + O_2$

(ii) Metabolic activation of organic compounds in the lung, liver and placenta induces expression of cytochrome P450 enzymes (CYP1A1) that generates ROS and reactive PAH-quinones.

(iii) Organic compounds contain quinone radicals that may undergo redox-cycling, producing hydroxyl radicals.

(iv) Macrophages and neutrophils phagocytose poorly soluble particles (metals, pollen, carbon core) that are deposited on the surface of epithelial cells in the airways and initiate an inflammatory response in the lung. Activated inflammatory cells are capable of generating ROS and reactive nitrogen species in order to remove foreign compounds.[12,55]

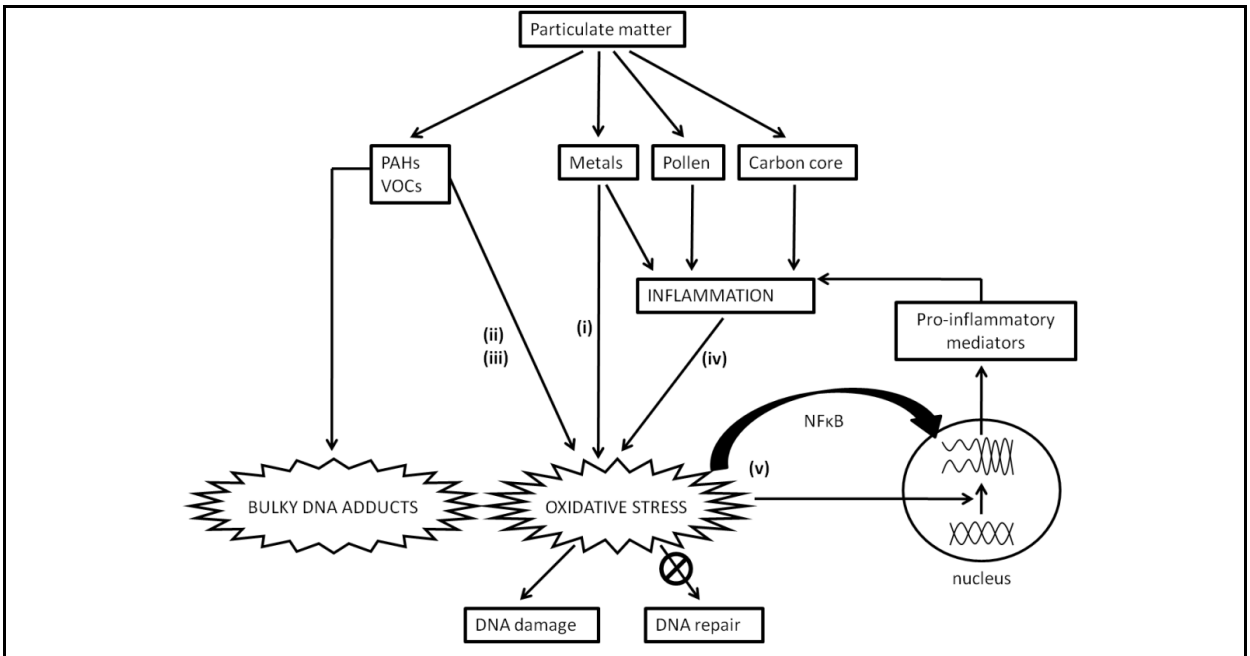


Figure 2. Molecular effects of exposure to particulate matter. PM induces oxidative stress that may arise from mixed sources via various reactions including Fenton reactions (i), metabolism (ii), redox-cycling (iii) and inflammation (iv). Activated inflammatory cells (macrophages, neutrophils) produce ROS to try to remove foreign material and restore redox balance. Contrary, a feedback mechanism involving NFκB induces transcription of pro-inflammatory mediators (cytokines) and proteins (antioxidants) that act to remove oxidative stress (v). The changed redox balance loosens histones from DNA, allowing transcription to occur. NFκB is an oxidative stress-responsive factor that links oxidative stress and inflammation. Oxidative stress damages DNA and metals such as Ni and Cr may inhibit NER[56]. In addition to ROS production, PAHs and volatile organic compounds (e.g. benzene) may be metabolically active and form bulky DNA adducts. Nuclear factor kappa B (NFκB); nucleotide excision repair (NER); Polycyclic hydrocarbons (PAHs).

Contrary to inflammation-induced oxidative stress, a feedback mechanism of oxidative stress contributes to the production of pro-inflammatory mediators (v) (Figure 2). Upregulation of pro-inflammatory mediators in response to transition metals were found to contribute to pulmonary inflammation. Also, organic compounds elicit inflammation through CYP1A1-mediated ROS generation, resulting in activation of transcription factors and release of cytokines[54]. Taken together, the hierarchical cellular oxidative stress model provides a mechanistic platform by which PM exert adverse health effects.

1.4 Markers of effect

Because air pollution is a mixture of many potential toxins, a decision must be made about which marker of effect to measure. In this study, the effects of PM on mitochondrial DNA are investigated.

1.4.1 Mitochondrial DNA

Mitochondria are intracellular organelles that are essential for cellular energy provision through the production of ATP via oxidative phosphorylation. Mitochondria also play a critical role in calcium storage, oxidant signalling, apoptosis, regulation of cell proliferation and metabolism. Each cell contains approximately 200 to 2000 mitochondria, each carrying 2 - 10 copies of mitochondrial DNA (mtDNA). The human mtDNA is a double stranded, circular molecule of 16.6 kb and contains 37 genes, encoding 13 proteins that are essential for oxidative phosphorylation and ATP production. Intra-mitochondrial synthesis of these proteins requires mtDNA-encoded 12S and 16S rRNAs and 22 tRNAs for their translation.[57,58] (Figure 3).

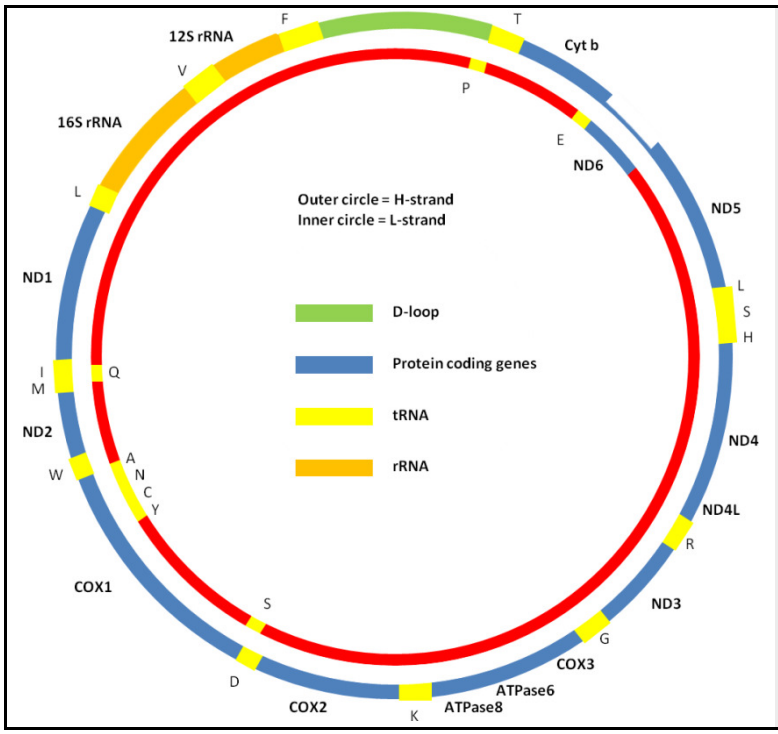


Figure 3. Structure of the human mitochondrial genome (16 569 bp). The genome consists of the Heavy-strand (outer circle) and the Leading-strand (inner circle). The majority of genes lie on the H-strand. Yellow boxes represent the genes coding for 22 tRNAs and the associated single letter codon refers to the amino acid. The two rRNAs (12S and 16S) and 13 polypeptides (ND, COX, ATPase and Cyt b) are depicted by orange and blue boxes respectively. The D-loop (green box) is a DNA triple helix with two copies overlapping the H-strand. It contains the origin of replication and place where promoters initiate transcription of the H-strand. NADH dehydrogenase (ND); Cytochrome c oxidase (COX); F_1F_0-ATP synthase (ATPase); Cytochrome b (Cyt b)

The major difference between human nuclear DNA (nDNA) and mtDNA is that the latter lacks protective histones, chromatin structure and introns. Additionally, the proof reading apparatus works less efficient than that of nDNA. Mitochondria are the major intracellular sources and primary targets of ROS.[59] In normal aerobic metabolism (ATP production), leakage of electrons from the mitochondrial respiratory chain leads to $O_2^{\bullet -}$ formation. As a consequence of the above listed factors, mtDNA is particularly vulnerable to ROS-induced damage and has a high mutation rate[59,60]. Mutations in the mtDNA occur more frequently in NADH dehydrogenase subunit 4 gene (ND-4) and in the displacement-loop (D-loop) region (Figure 3). The D-loop is a non-coding area that controls replication and transcription of mtDNA. It contains the site of origin of replication and the place where promoters initiate transcription. Consequently, the rate of mtDNA replication is affected by mutations in this region.[57]

mtDNA harbouring deleterious mutations are preferentially amplified as a compensatory response to energy deficiency by making more mitochondria and mtDNA copy numbers (mtDNAcn). In doing so, the mtDNA-content, referred as the copy number ratio of mtDNA to nDNA, increases. However, in time, the bio-energetic and replicative function of defective mitochondria declines and results in a decrease of mtDNAcn and thus mtDNA content.[59,61] In addition, mutations in nuclear genes involved in mtDNA synthesis or maintenance can affect mitochondrial biogenesis and therefore mtDNAcn[62]. In principle, the mtDNA-content can serve as marker for mitochondrial function and quantitative alterations of these mtDNAcn is important for understanding many cellular processes. Recently, mitochondrial function is linked to various disease mechanisms resulting from toxic exposures. UFP's have been shown to damage the inner membrane of mitochondria and disrupt mitochondrial cristae[55]. Quantitative alterations in mtDNAcn not only play a role in different diseases including multiple sclerosis[63], type II diabetes[64] and breast cancer[65] but also leads to abnormalities in birth weight[43]. It is necessary to understand the environmental interaction with mtDNA-content and its relation to disease.

1.5 Hypothesis

Air pollution-induced health effects during foetal and early postnatal life have been supported by numerous epidemiological studies[39-42] and reviews[36,66]. Studying newborns might be very relevant as *in utero* effects have been linked to the development of chronic diseases in adult life[48]. To date, mechanisms by which PM exposure may affect mitochondrial function are poorly understood. Biomarkers of mitochondrial function might be important in disease prediction and its association with toxic exposures has been barely studied. Research with placenta tissue is relatively new on this topic and this organ may serve as a biomarker of effect as represented by monitoring mtDNA-content. The exact mechanisms how PM exert its effects are not fully understood and requires considerable further research effort. In this cross-sectional study we hypothesize that mtDNA-content (i.e. relative mtDNAcn) of placental tissue changes in response to PM_{10} exposure during *in utero* life and may underlie susceptibility to mitochondrial dysfunction.

2 Material and methods

2.1 Study population and data collection

We recruited 178 mother-newborn pairs from Ziekenhuis Oost-Limburg (ZOL) in Genk between the 2nd of February 2010 and the 2nd of April 2011. Samples of placental tissue and umbilical cord blood were collected immediately after delivery, along with other clinical parameters such as birth data, sex, birth weight and length, gestational age (35 - 42 weeks), apgar score and ultrasonographic data including head circumference (HC), abdominal contour (AC), femur length (FL), biparietal diameter (BPD), transcerebral diameter (TCD), occipital-frontal diameter (OFD). All neonates were healthy at delivery and the apgar score after 1 min ranged from 2 to 10 but improved up to values between 7 and 10 after 5 min. The apgar score assesses the condition of a newborn and focuses on five objective clinical signs: skin colour (Appearance), pulse rate (Pulse), reflex irritability (Grimace), muscle tone (Activity) and breathing (Respiration). All mothers filled in a validated questionnaire to obtain detailed information on age, socioeconomic status, ethnicity, smoking, place of residence, medication, pre-gestational BMI, and parity. Socioeconomic status was coded and condensed into a scale with scores ranging from 1 to 3, on the basis of education. Ethnicity was assessed by looking at the native country of the infant's grandparents and was condensed into Europeans when two or more grandparents were European and into non-Europeans when at least three grandparents were from non-European origin. Current smokers were defined as having smoked during pregnancy. Before smokers were defined as those who quitted prior to pregnancy and never smokers did not smoke in their entire life. Birth data was condensed into a season scale where a difference was made between cold periods (October to March) and warm periods (April to September).

The study was conducted according to the principles outlined in the Helsinki declaration for investigation of human subjects. Written informed consent had been obtained from all study participants, in accordance with the procedures approved by the Ethical Committee of Hasselt University and ZOL.

2.2 Sample collection

Placental tissue and umbilical cord blood samples were processed in the laboratories of Hasselt University. A complete set of specimens was not available from all participants.

2.2.1 Blood collection

Umbilical cord blood was collected immediately after delivery in BD Vacutainer® Plus Plastic K_2EDTA Tubes. Samples were centrifuged at 3200 rpm for 15 minutes and instantly frozen, first at -20°C and afterwards at -80°C, to retrieve buffy coats for DNA extraction.

2.2.2 Placental sampling

174 placentas were obtained in the delivery room of ZOL in Genk and frozen within 15 minutes at -20°C. Tissue samples were collected following a standardized protocol as described in Adibi et al.[67] Briefly, villous tissue, protected by a foetal membrane, was biopsied from the foetal side of the placenta and preserved at -80°C. Each biopsy was taken approximately 4 cm away from the umbilical cord and 1 - 1.5 cm below the foetal membrane. Homogenization of the sampling was achieved using a device to orientate the foetal side of the placenta in relation to the umbilical cord. Care was taken to avoid foetal membrane contamination and to reach an amount of approximately 30 mg tissue.

2.3 Exposure measurement

PM_{10} is one of several parameters of ambient air quality that are continuously measured by a dense network of monitoring stations in Belgium.

2.3.1 Exposure to ambient particulate matter

Participant's home addresses were associated with coordinates (latitude/longitude) using geocoding. Regional background levels of PM_{10} were calculated using a model that provided interpolated PM_{10} values from the Belgian telemetric air quality networks in 4 km by 4 km grids. In order to explore potentially critical exposures during pregnancy, individually PM_{10} concentrations ($\mu g/cm^3$) were calculated for twelve subsequent periods: 0 - 7 days (Lag 0 - 7) before delivery, last month of pregnancy and for the three trimesters of pregnancy. Three trimesters were defined within the duration of pregnancy as: 1 - 13 weeks (Trimester 1), 14 - 28 weeks (Trimester 2) and 29 weeks to delivery (Trimester 3). Also, whole pregnancy expsoure was calculated as the mean of three trimesters. The date of conception was estimated based on ultrasound data and on the mother's last menstrual period.

Apparent temperature was measured each day, from start to end date of the study. The data was collected with usage of geocoding by the Koninklijk Meteorologisch Instituut (KMI) in Brussels.

2.3.2 Distance to major roads

Distances from home addresses to major roads were calculated through geocoding and is considered as a surrogate of traffic-related air pollution[68]. A distinction was made between an N-road (major traffic road) and an E-road (motorway/highway). The shortest distance was 10 meters, taken into account the space between actual residence and major road.

2.4 Quantitative analysis of mitochondrial DNA-content

Various toxic substances that damage mtDNA are partially responsible for a decrease in mitochondrial function. mtDNA-content serves as a marker for mitochondrial function and is referred as the mtDNAcn relative to single-copy gene nDNA copy number (nDNAcn). DNA was extracted from placental tissue and cord blood, using a new device based on a recent magnetic bead technology.

2.4.1 MagMAX™ Express 96 magnetic particle processor

The MagMAX™ Express 96 magnetic particle processor is designed for automated, high-throughput purification of high-quality nucleic acids from diverse sample matrices including fresh or frozen tissue, blood or buffy coat, mammalian cell cultures and buccal samples.

Nucleic acids are purified in a sequence of separate processes. Samples are treated with an appropriate lysis buffer to solubilise cellular membranes. After homogenization, samples are mixed with magnetic beads that have a nucleic acid binding surface. The nucleic acids bind to the beads and are magnetically captured. Several washing steps are included to remove cell debris, proteins and other contaminants (Figure 4). According to the desired nucleic acid, the mixture is treated with an appropriate nuclease. The purified nucleic acids are concentrated in a low salted elution buffer and can be used in a broad range of molecular applications.

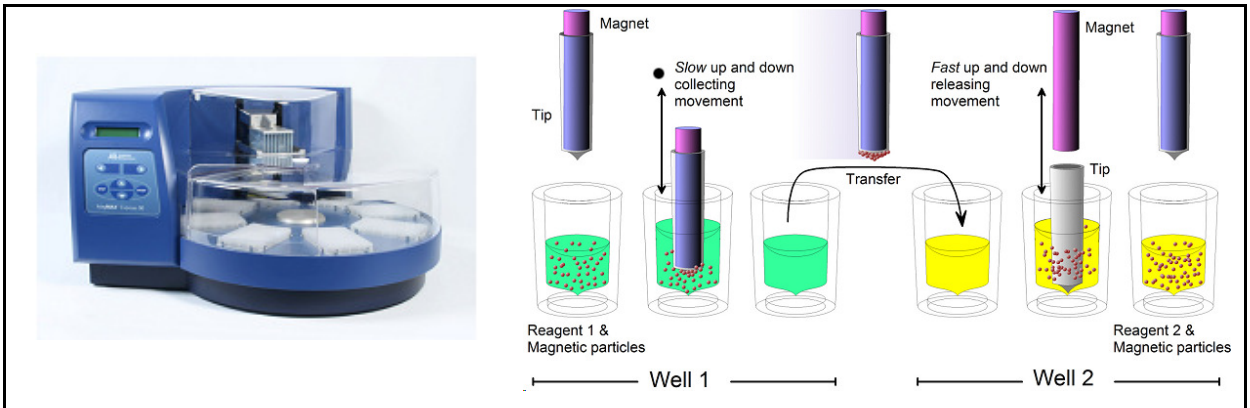


Figure 4. MagMAX™ Express 96 magnetic particle processor. A schematic view of magnetic particle processing.[69]

2.4.2 DNA extraction

Plasma was removed from thawed samples and buffy coats were collected prior to DNA extraction. DNA was extracted from white blood cells of the buffy coat and placental tissue cells using the MagMAX™ DNA Multi-Sample kit (Applied biosystems, Foster City, CA, USA) following the manufacturer's instructions. Briefly, this purification kit uses MagMAX™ magnetic bead-based nucleic acid isolation technology for producing high quantities of purified DNA. RNA contamination

was minimized with a RNase digestion step. The concentration of extracted DNA was measured at 260 nm with the Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, NE). Both DNA yield (ng/µl) and purity ratios (A260/280 and A260/230) were determined. Extracted DNA was stored at -20°C until further use.

2.4.3 qPCR primer design

After retrieving the human mitochondrial and nuclear genome from the NCBI databank, new primers were searched. Both mitochondrial and nuclear primers were identified using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), according to the criteria described in Table 1. The selected criteria were specific and optimized for our Fast SYBR® Green I dye in combination with the 7900HT Fast Real-Time PCR System. Afterwards, primer pairs were confirmed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and care was taken to avoid amplification of human genomic pseudogenes that were homologous to mitochondrial genes. mtDNA-content was assessed by comparing the average copy numbers of mitochondrial genes relative to the average copy numbers of nuclear genes.

Table 1. Criteria for selecting primers with Primer3.

	Minimum	Optimal	Maximum
Product size	85 nt	100 nt	115 nt
Primers size	18 nt	20 nt	22 nt
Primer Tm	55°C	60°C	65°C
Primer GC content	20 %	50 %	80 %
Max 3' self-complementarily	0		

The length of primer size was optimal for annealing to specific DNA sequences without annealing to human genomic pseudogenes. Small, specific primers were also less prone to primer dimer formation. The primer GC-content was associated with the primer melting temperature and used to verify the length of product that was formed at the end of the PCR reaction. Nucleotides (nt); Melting temperature (Tm).

2.4.4 Real-time quantitative PCR

Real-time quantitative Polymerase Chain Reaction (qPCR) has the ability to monitor the amplification and quantification of DNA and RNA in 'real-time' using a fluorescent reporter (i.e. Fast SYBR® Green I dye). SYBR Green detects PCR reaction products by binding to double-stranded DNA in a non-specific sequence manner. During every cycle of the PCR, AmpliTaq® Fast DNA-polymerase amplifies the target sequence and SYBR Green binds to each new copy of double stranded DNA. The increased fluorescent intensity is proportionate to the amount of formed DNA products. The cycle threshold (C_T)-value can be determined as the fractional cycle number at which the intensity passes a fixed fluorescence threshold. Consequently, the initial amount of DNA in the sample can be quantified and is inversely related with the C_T-value. Three phases can be distinguished in a qPCR reaction:

Exponential phase:	Accurate doubling of DNA
Linear phase:	Reaction slows down
Plateau phase:	Exhaustion of PCR products

The initial amount of DNA is quantified in the exponential phase which, after log-transformation, is transformed into a linear phase. The C_T-value is determined in the middle of this linear piece of the curve and represents the number of cycles needed to exceed the fixed threshold.

Extracted genomic DNA was diluted to a concentration of 5 ng/µl in RNase free water, prior to the qPCR runs. PCR reactions were set up by aliquoting 7.5 µl master mix into each well of a MicroAmp® Fast Optical 96-Well Reaction Plate compatible with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), followed by 2.5 µl of each experimental DNA sample, for a final volume of 10 µl per reaction. The master mix consisted Fast SYBR® Green I dye 2x (Applied Biosystems, Lennik, 5 µl/reaction), forward (0.3 µl/reaction) and reverse (0.3 µl/reaction) primer and RNase free water (1.9 µl/reaction). Primers were diluted to a final concentration of 300 nM in the master mix. Two non-template controls (NTCs) and six inter-run calibrators (IRCs) were carried along in each PCR plate. The thermal cycling profile was the same for all transcripts: 20 s at 95°C for activation of the AmpliTaq Gold® DNA-polymerase, followed by 40 cycles of 1 s at 95°C for denaturation and 20 s at 60°C for annealing/extension. Amplification specificity and absence of primer dimers were confirmed by melting curve analysis at the end of each run (15 s at 95 °C, 15 s at 60 °C, 15 s at 95 °C).

After thermal cycling, raw data was collected and processed. C_T-values of two mitochondrial genes were normalised relative to three reference genes according to the qBase software (Biogazelle, Zwijnaarde, BE). Therefore, mtDNA-content is a relative and dimensionless value. qBase is a flexible and open source program for qPCR data management and analysis[70]. The program uses modified software from the classic comparative C_T method ($\Delta\Delta C_T$) that takes multiple reference genes into account and uses inter-run calibration algorithms to correct for run-to-run differences. Using three reference genes, one needs to calculate a normalisation factor, i.e. the mean of the nuclear genes, to determine the mtDNAcn/nDNAcn ratio. Normalization is necessary to correct for forestalment fluctuations due to changes in concentration or volume. Inter-run calibration is required to calculate a calibration factor to remove technical run-to-run variation between samples analyzed in different runs due to instrument related variation (PCR block, lamp, filters, detectors), data analysis settings (baseline correction and threshold), reagents and optical properties of plastics. Calibration with multiple IRCs gives more precise results with a smaller error[70]

2.5 Statistical analysis

We used SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA) for database management and statistical analysis, and Graphpad Prism (version 5.0, Graphpad Software Inc., La Jolla, CA, USA) for figure design. Continues data were checked for normality and presented as arithmetic mean ± SD or geometric mean with interquartile range. Data not normally distributed were log-transformed. Categorical data were presented as frequencies (%) and numbers. Pearson's coefficient and linear regression was used to assess the relationship between mtDNA-content from placental tissue, umbilical cord blood and PM_{10} exposure. Multiple linear regression was performed to determine the independent variables of mtDNA-content. Covariates considered for entry in the model were maternal age, pre-gestational BMI, net weight gain, socioeconomic status, ethnicity, smoking status, medication, parity, infant's sex, birth weight and length, gestational age, season period and apparent week temperature. The multivariate model was determined by univariate analysis of relevant covariates and entered the model at $p \leq 0.05$. Maternal age, sex, smoking status, gestational age and ethnicity were forced into the model regardless of the p-value. Logical interactions were tested between covariates and accepted in the model at $p \leq 0.05$. We report results of unadjusted analyses (correlation figures) and results of fully adjusted models. For PM_{10} exposure, we estimated a change in mtDNA-content for each 10 $\mu g/cm^3$ increase in exposure.

3 Results

3.1 Characteristics of the study population

This cross-sectional study included 178 mother-newborn pairs, particularly from the middle region of Limburg in Belgium. Table 2 summarizes the maternal and infant's characteristics.

Maternal age ranged from 17 to 42 years and most women gave birth between age 20 and 29 (49.4 %). Most mothers were classified in the highest socioeconomic status (53 %). In those who smoked before pregnancy, 25 mothers continued smoking during pregnancy. The average pack-years for participants who smoked before pregnancy was 6.13 (± 5.08), but for the entire study population 1.5 (± 3.50). Prepregnancy BMI (24.3 kg/m^2 ± 4.79) had no influence on the newborn's weight ($p = 0.43$) or placental mtDNA-content ($p = 0.73$). For 84 women, this was the first delivery. Seven infants were preterm delivered (less than 37 weeks). The study population included 82 male and 96 female newborns with predominantly an European origin. Mean birth weight was 3403 g (± 386.66) and was in accordance with data from the Study centre for Perinatal Epidemiology in Flanders (3341 g)[71]. Mean birth weight was higher in boys than in girls (3514 g vs. 3309 g; $p = 0.0004$). Ultrasonographic data was collected during pregnancy week 30 and represents the intra-uterine growth. No complications were observed during intra-uterine growth. The apgar score improved the second time it was assessed. We determined the mtDNAcn/nDNAcn ratio in cells from placental tissue and cord blood of 174 and 176 subjects respectively.

Table 3 shows the outdoor exposure to PM_{10}, estimated during 0 to 7 days before delivery, last month before delivery, each trimesters of pregnancy and whole pregnancy. Average levels of PM_{10} exposure varied between 7 – 66 $\mu g/cm^3$ with a geometric mean of 21.50 $\mu g/cm^3$ for mean lag 0 – 7 and 11 - 58 $\mu g/cm^3$ with a geometric mean of 24.29 $\mu g/cm^3$ for the last month. Average trimester-specific PM_{10} exposure ranged from 13 - 37 $\mu g/cm^3$ with a geometric mean of 21.10 $\mu g/cm^3$ for the first trimester, 9 - 54 $\mu g/cm^3$ with a geometric mean of 38.91 $\mu g/cm^3$ for the second trimester and 14 - 43 $\mu g/cm^3$ with a geometric mean of 23.71 $\mu g/cm^3$ for the third trimester. Exposure for the whole pregnancy was 9 – 39 $\mu g/cm^3$ with a geometric mean of 28.07 $\mu g/cm^3$. The average distance from the participant's home address to nearest road was 207 m (range, 10 - 3960 m).

Table 2. Study population characteristics (n = 178).

	Mean (SD) or number (%)
Maternal characteristics	
Age, y	29.1 (± 4.92)
< 20	6 (3.4 %)
20-29	88 (49.4 %)
30-35	65 (36.5 %)
≥ 35	19 (10.7 %)
Socioeconomic status[a]	
Low	26 (15.6 %)
Middle	52 (31.1 %)
High	89 (53.3 %)
Smoking[b]	
Never	121 (71.6 %)
Before pregnancy	48 (28.4 %)
During pregnancy	25 (14.8 %)
Cigarettes / day	
0	124 (75.6 %)
1 to 10	28 (17.1 %)
≥ 11	12 (7.3 %)
Pack-years[b]	1.5 (± 3.50)
Medication[a]	107 (64.5 %)
Prepregnancy BMI, kg/m^2	24.3 (± 4.79)
Net weight gain, kg	14.5 (± 6.49)
Parity	
1	84 (47.2 %)
2	66 (37.1 %)
≥ 3	28 (15.7 %)
Infant's characteristics	
Gestational age, w	39.2 (39 - 40)
Preterm delivery, < 37 w	
Yes	7 (3.9 %)
No	171 (96.0 %)
Sex	
Male	82 (46.0 %)
Female	96 (53.9 %)
Ethnicity[b]	
European	149 (88.2 %)
Non-European	20 (11.9 %)
Season	
Cold period	104 (58.4 %)
Warm period	74 (41.6 %)
Apgar score	
1 min	8.4 (8 - 9)
5 min	9.5 (9 - 10)
Neonate birth weight, gr	3403 (± 386.66)
Neonate length, cm	50.2 (49 - 51)
Ultrasonographic data w30, mm[c]	
Head circumference	284 (276 - 292)
Abdominal contour	262 (251 - 273)
Femur length	58 (± 2.95)
Biparietal diameter	81 (± 4.28)
Occipital-frontal diameter	100 (97.3 - 102.9)
Transcerebral diameter	36 (± 2.66)
Placenta (mtDNA/nDNA)[d]	1.028 (0.64 - 1.63)
Umbilical cord (mtDNA/nDNA) [e]	1.019 (0.75 - 1.30)

Data are presented as arithmetic means ± standard deviation (SD) or number (%). Not normally distributed values are presented as geometric means with 25 - 75 percentiles. Mitochondrial DNA (mean of MTF3212/R3319 and ND-1) is normalised to nuclear DNA (mean of 36B4, ND-1 and HBG-1).

[a] Data available for 167 subjects. [b] Data available for 169 subjects. [c] Data available for 170 subjects. [d] Data available for 174 subjects. [e] Data available for 176 subjects.

Table 3. Outdoor exposure characteristics for the study population.

	Geometric mean	Min	Percentile			Max	IQR
			25 %	50 %	75 %		
Particulate matter							
Lag 0	20.30	6.2	15.2	20.6	26.5	64.0	11.3
Lag 1	20.31	5.9	15.1	19.7	26.9	71.8	11.8
Lag 2	20.72	4.3	14.6	20.8	28.8	112.0	14.4
Lag 3	22.67	5.1	14.8	21.4	31.9	107.1	17.2
Lag 4	23.12	6.4	15.4	21.6	32.8	130.4	17.4
Lag 5	22.01	5.9	15.8	21.5	30.3	117.0	14.6
Lag 6	21.28	5.4	15.8	20.8	27.7	104.8	12.0
Lag 7	21.76	5.5	15.0	20.4	29.8	138.5	14.8
Mean Lag 0-7	21.50	7.2	16.8	20.6	28.1	65.9	11.2
Last month	24.29	11.3	19.3	23.8	29.5	57.7	10.2
Trimester 1	21.10	13.5	18.1	20.7	23.7	36.9	5.6
Trimester 2	38.91	9.5	35.4	38.5	43.1	53.9	7.7
Trimester 3	23.71	13.6	20.2	23.9	28.1	42.6	7.9
Whole pregnancy	28.07	9.5	25.1	27.8	31.2	39.0	6.1
Distance to major roads							
Nearest major road	207	10.0	85	352.5	676	3960	590.6
Mean distance N-road	380	10.0	87	355.0	772	3960	685.0
Mean distance E-road	3052	32.86	1950	3660	5780	17460	3830

The average PM_{10} concentration ($\mu g/cm^3$) is represented for twelve subsequent periods: 0 - 7 days (Lag 0 - 7) before delivery, last month of pregnancy and for the three trimesters of pregnancy. PM_{10} exposure during whole pregnancy is represented as the mean of trimesters. Distance to major road is measured in meters. Nearest major road does not distinguish between N- or E-roads.
Minimum (Min); 25 percentile (25 %); Median (50 %); 75 percentile (75 %); Maximum (Max); Interquartile range (IQR).

3.2 Validating methods for qPCR

Primer pairs were selected with Primer3 and confirmed with BLAST. Afterwards, each primer pair was tested to assess their efficiency and specificity. Relative mtDNA-content was measured by determining the ratio of two mtDNAcn to three nDNAcn. The three reference single-copy genes used in this study were 36B4, HBG-1 and β-act and the two mitochondrial genes were MTF3212/R3319 and ND-1. In Table 4, accession numbers and annotated sequences of primer pairs are shown.

Table 4. Selected genes with their accession number and annotated sequence.

Name	Accession number	Reference/ Mitochondrial	Forward 5' - 3'	Reverse 5' - 3'
MTF3212/R3319	NC_012920.1	Mitochondrial	CACCCAAGAACAGGGTTTGT	TGGCCATGGGTATGTTGTTAA
ND-1	NC_012920.1	Mitochondrial	ATGGCCAACCTCCTACTCCT	CTACAACGTTGGGGCCTTT
36B4	NT_009775.17	Reference	GGAATGTGGGCTTTGTGTTC	CCCAATTGTCCCCTTACCTT
β-act	NT_007819.17	Reference	ACTCTTCCAGCCTTCCTTCC	GGCAGGACTTAGCTTCCACA
HBG-1	NT_009237.18	Reference	GTGCACCTGACTCCTGAGGAGA	CCTTGATACCAACCTGCCCAG
GADPH	NT_009759.16	Reference	CCCCACACACATGCACTTACC	AATCAAAGCCCTGGGACTAGG

Mitochondrial forward primer from nucleotide 3212 and reverse primer from nucleotide 3319 (MTF3212/R3319); NADH dehydrogenase subunit 1 (ND-1); acidic ribosomal phosphoprotein P0 (36B4, also known as RPLP0); β-actin (β-act); haemoglobin gamma A (HBG-1); glyceraldehyde-3-phosphate dehydrogenase (GADPH).

3.2.1 Primer efficiency

Each primer pair was tested with a DNA serial dilution: $^1/_1$, $^1/_4$, $^1/_{16}$, $^1/_{64}$, $^1/_{256}$, $^1/_{1024}$, $^1/_{4096}$. This was necessary to see if primers work properly in both high and low DNA concentrations. The C_T-value with associated logarithmic concentration was plotted to obtain a standard curve (Figure 5). The slope of the standard curve was used to calculate the reaction efficiency with formula:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1.$$

Generally, an efficiency between 85 % and 115 % ($-3.6 \geq \text{slope} \geq -3.1$) and R^2 above 0.99 was considered acceptable. A reaction efficiency of 100 % (E = 1) meant an exact doubling of the product in the exponential phase.

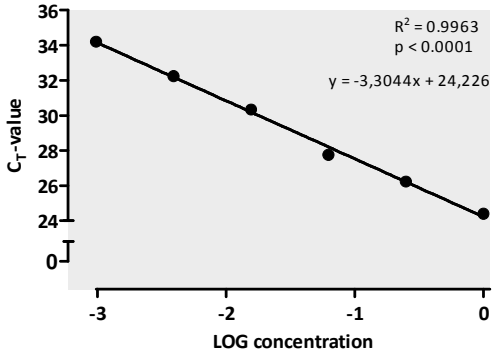


Figure 5. Example of a standard curve of the human nuclear reference gene (36B4) from placental tissue. The logarithmic concentration is plotted against the corresponding C_T-value. The slope is depicted in the equation of the linear regression line. The coefficient of determination (R^2) is a measure for the conformity between the standard curve and the data and lies between 0 and 1. It is a statistical term that says how well values are likely to be predicted by the model.

Each primer pair was tested in different DNA concentrations and efficiencies with R^2 are shown in Table 5. In the final PCR reactions, we used a DNA concentrations of approximately 5 ng/µl. High DNA concentrations (10 ng/µl) had amplification plots that deviated from amplification plots of the rest of the dilution serie. Baring this in mind, the two lowest values of the 6.3 ng/µl dilution series had been excluded because these amplification plots deviated also. Consequently, a DNA concentration of 5 ng/µl was the best option for the primer efficiency because this concentration fell right into this range. The reference gene GADPH was left out for the mtDNA analysis because the efficiencies from the standard curves were unreliable after repeated measurements.

Table 5. Efficiency and correlation coefficient of each gene.

	6.3 ng/µl*		9.5 ng/µl	
Name	**Efficiency (%)**	**R^2**	**Efficiency (%)**	**R^2**
MTF3212/R3319	96	0.9974	102	0.9938
ND-1	99	0.999	101	0.9992
36B4	100	0.9963	104	0.9982
β-act	97	0.9994	97	0.9989
HBG-1	100	0.9984	103	0.9982
GADPH	92	0.9895	98	0.9957

Two concentrations of DNA are given (6.3 ng/µl and 9.5 ng/µl).
* The two latter C_T-values of the dilutions from the 6.3 ng/µl standard curve are excluded.

3.2.2 Correlations of mitochondrial and nuclear genes

After processing the C_T-values with qBase, correlations were made between all nuclear genes and all mitochondrial genes separately to assess their relationship to each other.

Nuclear genes

Three nuclear genes are plotted against each other for placental tissue and cord blood separately (Figure 6). The correlation coefficient (r) and p-value are given for each plot. All three nuclear genes correlated well in the cord blood. Nevertheless, the HBG-1 gene correlated less in placental tissue than in cord blood. This observation was kept in mind in further analysis because the normalisation factor was based on the mean C_T-values of the nuclear genes.

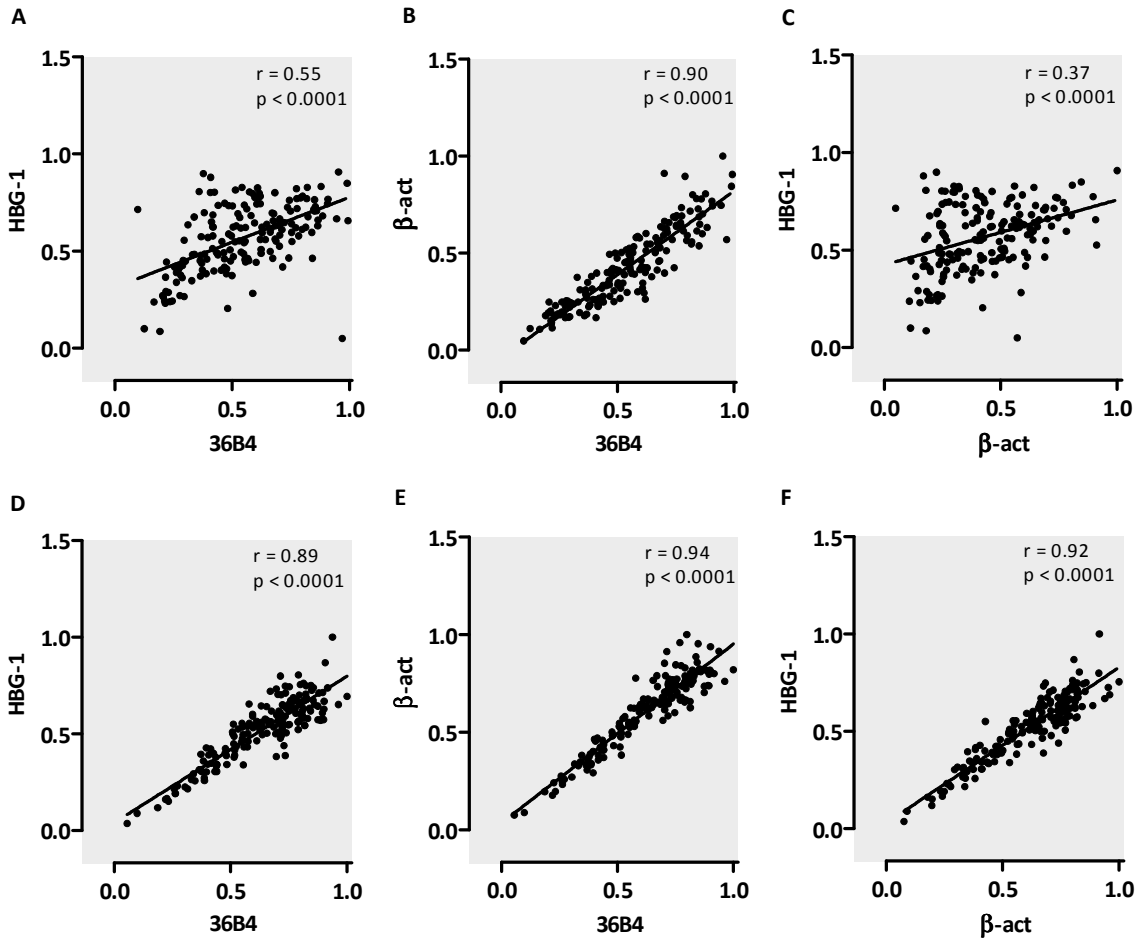


Figure 6. Correlations of nuclear reference genes. Nuclear genes from placental tissue are shown in the top row (A - C) and nuclear genes from umbilical cord blood in the bottom row (D - F). All values are non-logarithmic and scaled to the maximum value. Acidic ribosomal phosphoprotein P0 (36B4, also known as RPLP0); β-actin (β-act); haemoglobin gamma A (HBG-1).

Mitochondrial genes

Both mitochondrial genes (MTF3212/R3319 and ND-1) from placental tissue and cord blood correlated well (Figure 7). Outliers were present in both scatter plots and are indicated with a circle. Interestingly, those who deviate, had this deviation in both cell types and were subjects with Moroccan nationality. The C_T-values were normalized with a normalisation factor, derived from the average C_T-value of the three reference genes. Taking into account that the HBG-1 gene correlated less in placental tissue, a normalisation factor was calculated without the HBG-1 reference gene. This correlation plot deviated minimally from the original plot (data not shown). Further analysis was done with all three reference genes for normalisation.

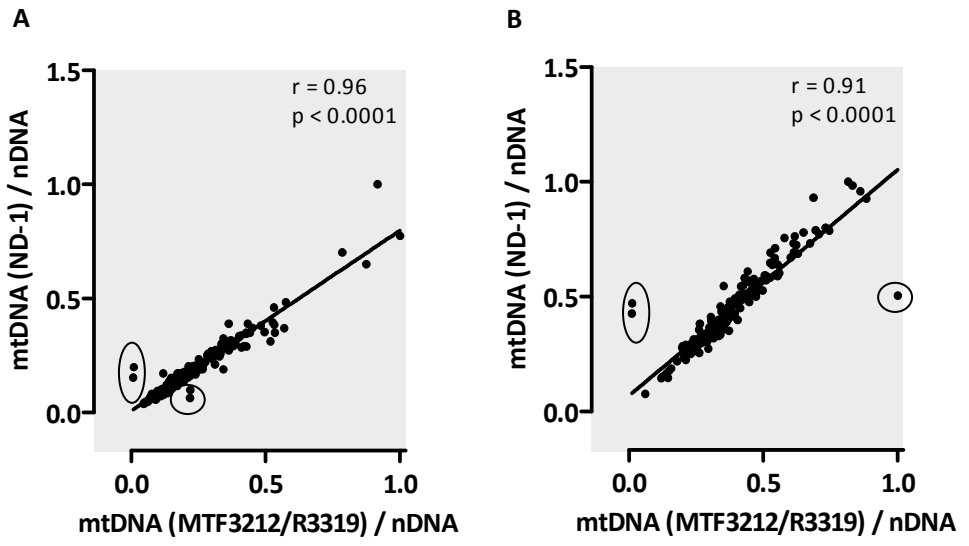


Figure 7. Correlation of two mitochondrial genes for placental tissue (A) and umbilical cord blood (B). Outliers are indicated with a circle. All values are non-logarithmic and scaled to the maximum value. Mitochondrial DNA was calculated relative to a normalisation factor that is the average of the three nuclear genes 36B4, HBG-1 and β-actin. Mitochondrial forward from nucleotide 3212 and reverse from nucleotide 3319 (MTF3212/R3319); NADH dehydrogenase subunit 1 (ND-1).

3.3 Comparison of mtDNA-content in placental tissue and cord blood

The relative mtDNAcn (ratio mtDNAcn/nDNAcn) was determined by the normalised C_T-values and reflected the relative mtDNA-content. In Figure 8A is shown that the mean normalized C_T-value of placental tissue (1.26 ± 0.067) differed from cord blood (1.12 ± 0.042), although this did not reach significance (p = 0.08). This observation was not attributable to a difference in nuclear reference genes. Mean nDNAcn of placental tissue (1.068 ± 0.027) was not different compared with nDNAcn of cord blood (1.079 ± 0.024). This showed that the nDNA-content in both these cell types was the same (p = 0.76). Furthermore, these two cell types did not correlate well (p = 0.11, Figure 8B), verifying a difference between the two cell types.

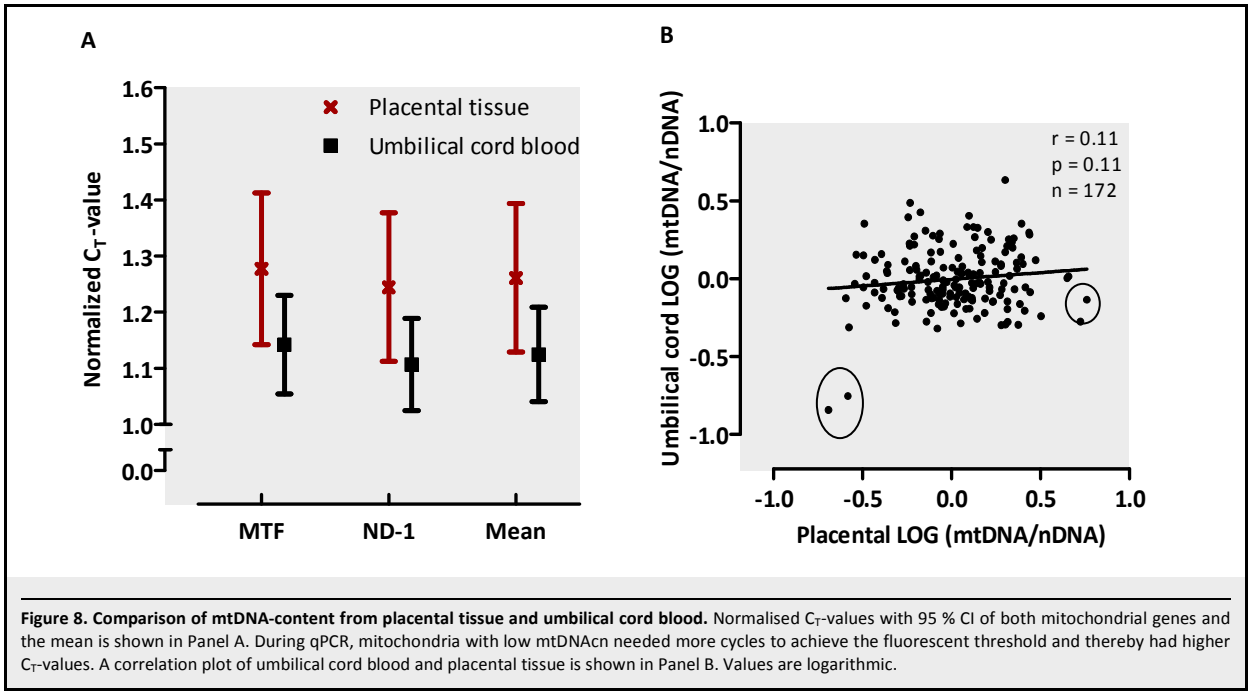


Figure 8. Comparison of mtDNA-content from placental tissue and umbilical cord blood. Normalised C_T-values with 95 % CI of both mitochondrial genes and the mean is shown in Panel A. During qPCR, mitochondria with low mtDNAcn needed more cycles to achieve the fluorescent threshold and thereby had higher C_T-values. A correlation plot of umbilical cord blood and placental tissue is shown in Panel B. Values are logarithmic.

3.4 Association between air pollution exposure and mtDNA-content

Univariate analysis showed that mtDNA was influenced by different predictors. Placental mtDNA-content was independently and positively associated with apparent week temperature (β = 0.0204 ± 0.003; p < 0.0001), negatively associated with cold season period (β = -0.243 ± 0.040; p < 0.0001) and negatively associated with parity (β = -0.064 ± 0.027; p = 0.0183). Further analysis revealed that there was a negative relationship between placental mtDNA-content and exposure to PM_{10} during the pregnancy. This association was statistically significant for whole pregnancy exposure (r = -0.15, p = 0.046), but more particularly for the last periods of pregnancy exposure (Figure 9). Unadjusted analysis showed that placental mtDNA-content was significantly correlated with PM_{10} exposure during the third trimester of pregnancy (r = 0.40, p < 0.0001), the last month of pregnancy (r = -0.46, p < 0.0001) and mean lag 0 - 7 (r = -0.34, p < 0.0001). In addition, the correlation was significant for each lag separately (data not shown). PM_{10} exposure during the first and second trimester of pregnancy were not significant (p = 0.31 and p = 0.67 respectively). In contrast, none of these subsequent periods correlated significantly with mtDNA-content from cord blood: Trim 1 (p = 0.99), trim 2 (p = 0.96), trim 3 (p = 0.54), last month (p = 0.58) and mean lag 0 - 7 (p = 0.96).

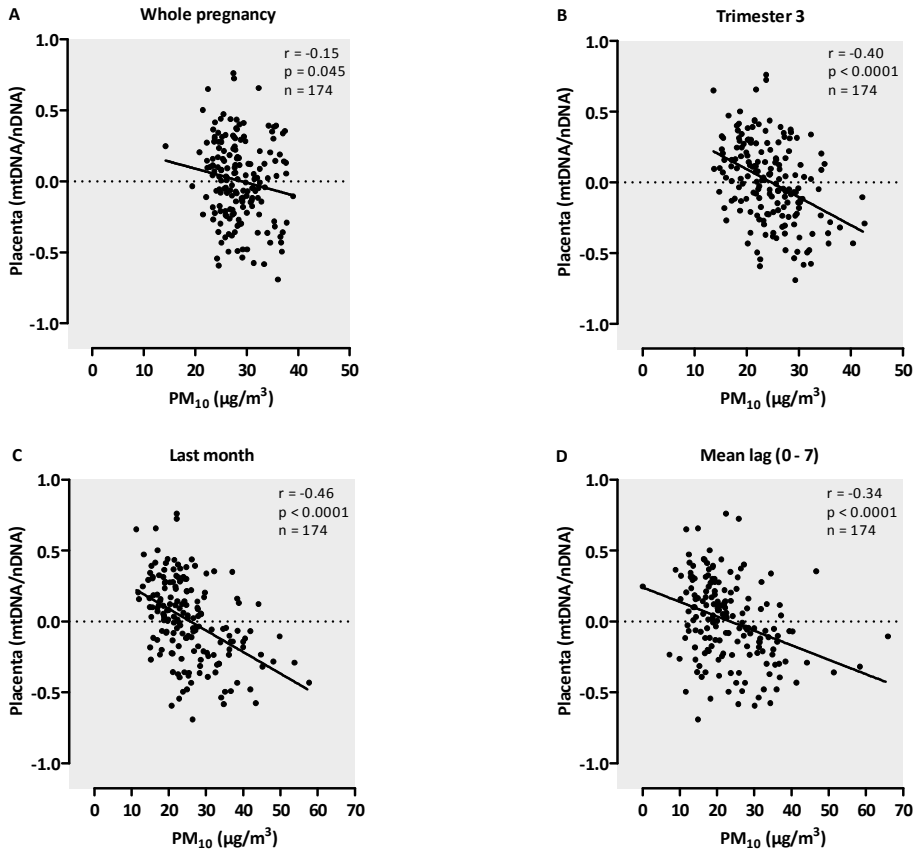


Figure 9. Correlation between PM_{10} exposure and mtDNA-content in placental tissue. Four correlation plots are given, each indicating PM_{10} exposure during a period of pregnancy: Whole pregnancy (A), third trimester (B), the last month of pregnancy (C) and mean of 7 days before delivery (D). PM_{10} concentrations are given in µg/cm^3. Values of placental mtDNA-content are logarithmic.

Additionally to PM_{10} exposure as solely predictor variable, we computed a multivariate model to adjust for relevant variables that may influence the mtDNA-content outcome. Although maternal age, sex, smoking status, gestational age and ethnicity were not significantly associated with placental mtDNA-content, we forced these variables into the regression model, together with parity, apparent week temperature and season period. After adjustment for the aforementioned variables, placental mtDNA-content remained negatively associated with PM_{10} exposure during the third trimester, last month, mean lag 0 - 7 and marginally associated with whole pregnancy exposure (Table 6).

Figure 10 shows a change in mtDNA/nDNA ratio of placental tissue for each 10 $\mu g/cm^3$ change of PM_{10} exposure. Each 10 $\mu g/cm^3$ increase in PM_{10} was associated with a lower placental mtDNA/nDNA ratio of β = -0.161 (95% CI: -0.094 to -0.229; $p < 0.0001$) in the third trimester, β = -0.113 (95 % CI: -0.064 to -0.162; $p < 0.0001$) in the last month and β = -0.053 (95 % CI: -0.007 to -0.098; $p = 0.0239$) in the mean lags (Table 6). Although this lower ratio was significant for the mean lag 0 - 7, lag 0 - 2 did not reach significance

More striking was the fact that a serious decrease in mtDNA/nDNA ratio was seen as subsequent trimesters pass by, indicating a potential window of susceptibility by trimester of exposure. mtDNA-content seemed to increase for each 10 $\mu g/cm^3$ increase in PM_{10} exposure in the first trimester by β = 0.064 (95 % CI: -0.015 to 0.143, $p = 0.11$). In the second trimester, no significant change was observed ($p = 0.26$). Yet, whole pregnancy exposure showed that this decrease persisted, although marginally significant (β = -0.081, 95 % CI: -0.173 to 0.010, $p = 0.08$).

In addition, newborns born in the cold season period had a lower relative mtDNAcn than newborns born in a warm period, even after adjustment of cofounding variables. This was significant during the late periods of pregnancy: Third trimester β = -0.144, 95 % CI: -0.256 to -0.037; $p = 0.0091$. The same observation was made with parity. Multiparous mothers generally had less mtDNAcn than primiparous mothers. Hence, in addition to PM_{10}, there was a significant effect of these parameters on mtDNA-content, however, the effect of PM_{10} exposure was most significant.

Figure 10 confirmed the findings seen in the correlation plots of mtDNA-content in cord blood. Neither pregnancy period was associated with a significant change in mtDNA/nDNA ratio for each 10 $\mu g/cm^3$ increase in PM_{10} exposure, although the 95 % confidence intervals indicate that lag 0 - 2 are marginally associated with a lower mtDNA-content in cord blood. Whereas placental tissue had a lower mtDNA-content during subsequent trimesters, the cord blood mtDNA-content remained the same.

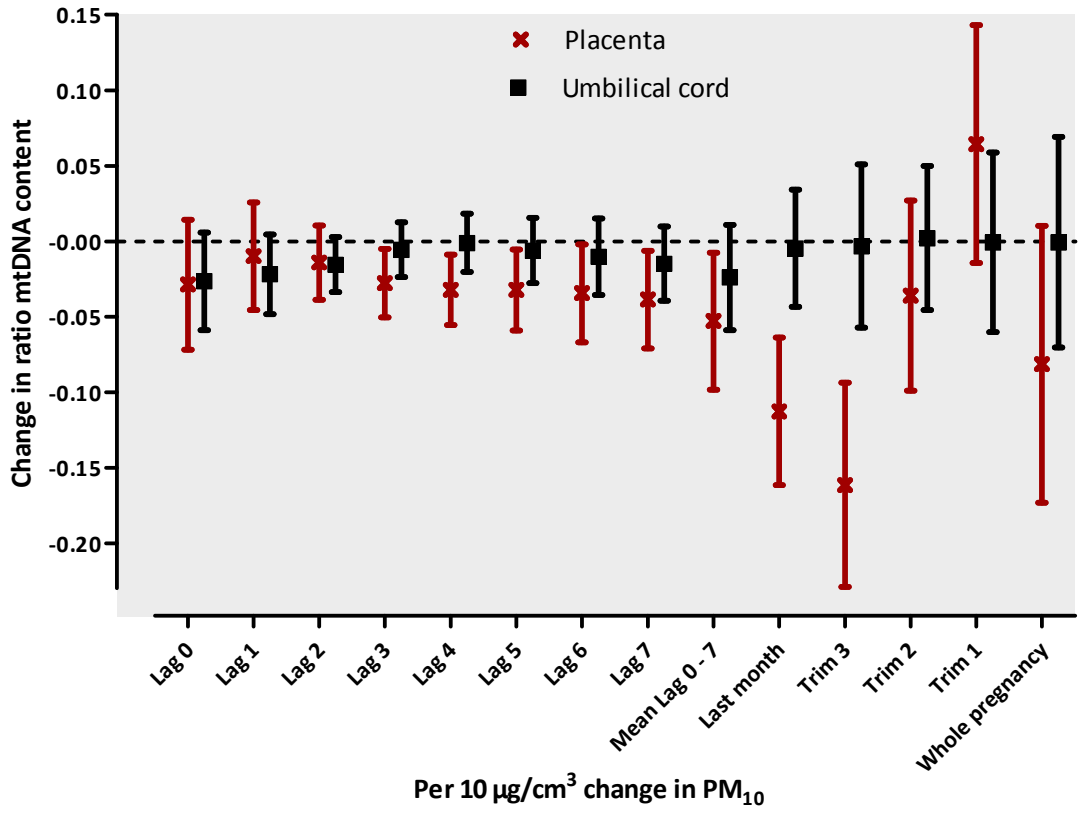


Figure 10. Change in mtDNA/nDNA ratio of both placental tissue and cord blood for each 10 µg/cm^3 increase of PM_{10} exposure. The relative mtDNAcn represents the mtDNA-content. The model is adjusted for maternal age, infant's sex, parity, gestational age, ethnicity, smoking status, season period and apparent week temperature. Calculations are made with logarithmic values. A decrease in mtDNAcn is significant when the 95 % CI lies under zero.

Table 6. Estimated change in placental mtDNA-content in association with PM_{10} during pregnancy or distance from residence to nearest major road.

PM_{10}‡

	Estimate	95% CI	P-value
Model 1*			
Trimester 3	-0.1979	-0.2667 to 0.1291	< 0.0001
Last month	-0.1520	-0.1965 to 0.1075	< 0.0001
Mean lag 0 - 7	-0.1019	-0.1444 to 0.0595	< 0.0001
Whole pregnancy	-0.0989	-0.1952 to 0.0026	0.0457
Model 2†			
Trimester 3	-0.1614	-0.2291 to 0.0934	< 0.0001
Last month	-0.1127	-0.1615 to 0.0639	< 0.0001
Mean lag 0 - 7	-0.0529	-0.0984 to 0.0074	0.0239
Whole pregnancy	-0.0814	-0.1731 to 0.0103	0.0838

Distance to nearest major road$

	% mtDNA/nDNA	95% CI	P-value
Model 1*			
	3.3	0.2 to 6.5	0.2844
Model 2†			
	5.8	2.9 to 8.8	0.0477

‡ Effect size was calculated for each 10 µg/cm^3 increase in PM_{10} exposure at each woman's residence during the corresponding period.
$ Effect size was calculated for each doubling in distance from residence to major road (based on a model with log distance and log mtDNA-content).
***** Unadjusted model.
† Adjusted for maternal age, infant's sex, parity, gestational age, ethnicity, smoking status, season period and apparent week temperature.

3.5 Markers of traffic-related air pollution

An association between placental mtDNA-content and PM_{10} exposure was demonstrated in the previous section. PM_{10} was used as an indicator of traffic-related air pollution. Alternatively, distance to major road is an exposure marker that was used as a surrogate for traffic-related air pollution[68]. The distances from the home address to major roads were calculated for each participant. The nearest distance was set at 10 meters, taken into account the space between the residence and road. The relation between PM_{10} concentration and distance to nearest major road is shown in Figure 11. Participants living close to a major road were exposed to higher concentrations of PM_{10}, although this negative correlation was not significant (p = 0.09). Nevertheless, after adjusting for maternal age, sex, smoking status, gestational age, ethnicity, parity, apparent week temperature and season period, this finding reached significance (p = 0.0447). This indicated that subjects who lived closer to a major road had a lower relative mtDNAcn in mitochondria of placental tissue. Each doubling in distance was associated with a significant increase in mtDNA/nDNA ratio by 5.8 % (95 % CI: 0.2 % to 6.5 %).

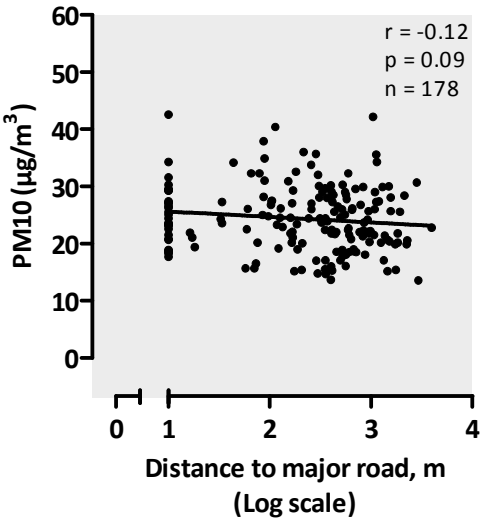


Figure 11. Distance to major roads as a marker of traffic-related air pollution. Correlation between PM_{10} concentration and distance to nearest major road. The nearest distance was set at 10 meters to account for the space between residence and road. PM_{10} concentration is given in $\mu g/cm^3$. Distance to major road is measured in meters. Values of distance to major road are logarithmic.

3.6 Other observations

Bearing in mind the importance of mitochondrial function in placental tissue, we hypothesized a relationship between possible adverse neonatal outcomes and changed placental mtDNA-content. Looking at birth weight and length of neonates, we did not found an association with mtDNA-content ($p = 0.53$ and $p = 0.45$ respectively). Even when we condensed infants into small-for gestational age and normal-for gestation age, we still not found a significant change ($p = 0.78$). No association was found between birth weight and cord blood mtDNA-content (data not shown).

In addition to the effects of PM_{10} on mtDNA-content of placental tissue, we sought an association between mtDNA-content of placental tissue and maternal smoking during pregnancy. This finding did not reach significance in the unadjusted analysis ($p = 0.23$). When we took only Europeans into account, and we adjusted for sex, maternal age, parity and gestational age, we observed that there was a marginally significant association between placental mtDNA-content and smoking during pregnancy ($p = 0.08$). Nevertheless, we found an association between smoking during pregnancy and birth weight. Infants born to smoking mothers were on average 230 g lighter at birth than infants of non smokers, even after adjustment for gestational age ($p = 0.0039$).

4 Discussion

In this cross-sectional study, we evaluated substantive changes in mitochondrial DNA-content related to PM_{10} exposure in placental tissue and cord blood of mother-newborn pairs. We found a significant association between exposure to PM_{10} during the third trimester of pregnancy and a lower relative mtDNAcn in placental tissue ($p < 0.0001$). Accordingly, a lower relative mtDNAcn was also seen in the last month ($p < 0.0001$) and the last week ($p = 0.0239$) of pregnancy. This association could not be explained by maternal age, sex, smoking status, gestational age, ethnicity, parity, season period and apparent week temperature or any other covariate studied. In contrast, none of the subsequent periods of pregnancy showed a change of mtDNA-content in cord blood. The mtDNA-content of placental tissue differed from that of cord blood and both cell types did not correlate well. ($p = 0.11$). We also noted an association between distance to major roads, a surrogate for traffic-related air pollution, and placental mtDNA-content ($p = 0.0447$).

4.1 Mechanisms by which air pollution affects mitochondrial function

Mitochondria have multiple, essential roles in cell function including energy production, oxidant signalling, apoptosis, immune response and thermogenesis[58]. Cell viability depends on these crucial processes and is maintained by proper mitochondrial function. The placenta is a highly metabolic active organ that plays a role in nutrient transfer, growth and organ development. These processes require a lot of energy and are regulated by mitochondrial function of placental cells. Taken together, placental mitochondria play an important role in the proper formation and functioning of the placenta and, therefore, are essential for foetal health. It is well known that urban particulate air pollution has adverse effects on the functional morphology of the placenta and health outcome of the foetus[38,40,42,43,66,72,73]. Also, environmental factors can damage mitochondrial DNA (mtDNA) and affect mitochondrial function. The biological mechanism by which air pollution may affect mitochondrial function is poorly understood[72]. Therefore, we can only speculate about possible mechanisms linking PM exposure and mitochondrial function. Aspects that may play an important role are oxidative stress, endothelial function and altered gene expression. (Figure 12)

Pregnancy is a state of oxidative stress in which increased metabolic activity in placental mitochondria and the reduced scavenging power of oxidants may be responsible for reactive oxygen species (ROS) generation in the placenta[74]. In addition, PM_{10} exposure also contributes to oxidative stress as indicated in Figure 12. PM-induced ROS are present in the mitochondria and cytoplasm of placental cells. At the same time, ROS is formed as a by-product in normal mitochondrial respiration.

Excessive ROS production by placental mitochondria injures intracellular constituents including DNA, RNA, lipids, proteins and mitochondrial cristae, impacting mitochondrial function[55]. mtDNA is particularly vulnerable to ROS-induced damage and has a high mutation rate. mtDNA harbouring mutations are preferentially amplified as a compensatory response to ATP deficiency.

On the other hand, hypoxia and deprivation of nutrients contribute to an ischemic energy deficiency in cells. The placenta is very sensitive in the first trimester of pregnancy to oxygen-mediated damage[74]. It has been shown that PM, particularly pro-oxidative combustion particles, influences endothelial function[73,75]. Both particle cores as well as adsorbed species induce cytokine production, systemic inflammation and inhibit nitric oxide vasodilator activity, resulting in endothelial dysfunction. Moreover, ROS are formed in mitochondria of endothelial cells that damages mtDNA. Endothelial ROS, together with vasoactive responses, mediate a PM-induced vasoconstriction, leading to compromised placental perfusion and hence, ischemia[37,38,76,77]. The hypoxia state of trophoblasts also contributes to the oxidative status. In addition to vasoconstriction, PM alters trophoblast differentiation leading to changes in placental development and vasculature [4-7]. Herein, peroxisome proliferator activated receptor protein gamma (PPARγ) plays an important role. PPARγ is a nuclear transcription factor that is essential in basic placental development and function through the regulation of genes involved in trophoblast differentiation, angiogenesis, fatty acid transport and inflammation[67]. An oxidative stress state may disrupt transcription of crucial genes and key signalling molecules, under the regulation of PPARγ, that are necessary for proper placental structure. PPARγ-coactivator alpha (PGC-1α) is a transcriptional coactivator of PPARγ, but also controls other nuclear receptors and transcription factors that are essential in mitochondrial biogenesis and energy metabolism. For example, an interaction with nuclear respiratory factor 1 stimulates synthesis of TFAM, which is crucial for mtDNA transcription and maintenance and, therefore, relates to mtDNA-content. PGC-1α is responsive to ROS, nutrient availability, hypoxia, ATP demand and provides a direct link between external physiological stimuli and regulation of mitochondrial biogenesis.[78,79] PM may have effects on the expression of this essential coactivator, resulting in changes in expression of PPARγ-dependent genes and genes controlling mitochondrial biogenesis and mtDNA-content of placental mitochondria.

Together with vasoconstriction, changes in structure and vasculature limit the placenta to mediate nutrient and oxygen transport[14,38,40,42,73,80]. These processes are regulated by systems that are dependent on the cellular formation of ATP. Hence, both energy deficiency and PM-induced mutations may have combined or separate effects on mitochondrial function. Mitochondria will synthesize more copies of their mtDNA (mtDNAcn) and increase their mitochondrial abundance to meet with energy deficiency[59]. This compensatory mechanism is part of a vicious circle resulting in

more ROS formation from defective cells and leads to damage of mitochondria. In time, the bio-energetic and replicative function of defective mitochondria declines and results in a lower relative mtDNAcn and thus mitochondrial dysfunction[61]. Taken together, oxidative stress and altered placental function are important mechanisms by which air pollution affects mitochondrial function.

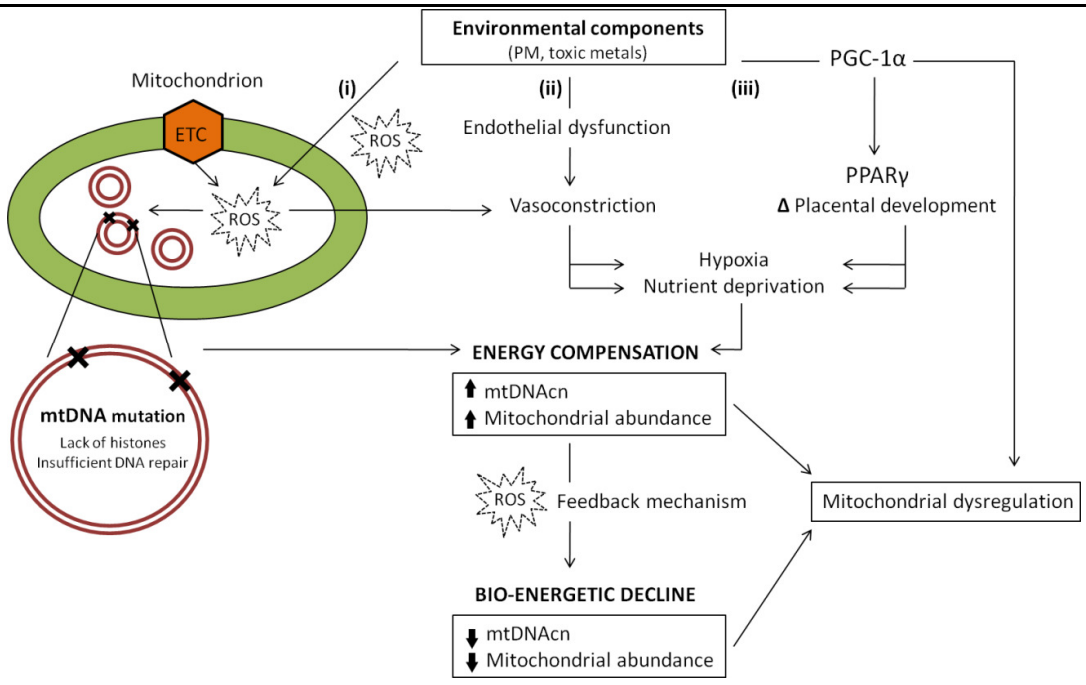


Figure 12. Hypothesized mechanisms of mitochondrial dysregulation by environmental components. Environmental components such as PM_{10} or transition metal constituents contribute to ROS generation (i). In addition to these sources, PM-activated inflammation cells generate ROS and RNS. mtDNA is particularly vulnerable to mutations due to the lack of protective histones and an insufficient DNA repair system. Combustion-derived particles contribute to endothelial dysfunction and leads to vasoconstriction (ii). This response, together with altered placental development (iii), lead to ischemic reactions in the placenta. An energy compensation, in response to mutations and ischemia, lead to a higher mtDNA-content. Ultimately, due to excessive ROS formation (feedback mechanism), the bio-energetic and replicative function of mitochondria declines. Both an increase and a decrease in mtDNA-content are associated with mitochondrial dysregulaton. Mitochondrial dysfunction may also be due to adverse effects of PM on PGC-1α. This coactivator is responsible for transcription of nuclear genes that control mitochondrial biogenesis and function. Reactive oxygen species (ROS); Reactive nitrogen species (RNS); Peroxisome proliferator activated receptor protein gamma (PPARγ); PPARγ coactivator α (PGC-1α).

4.2 mtDNA-content as a marker of effect

Mitochondrial dysfunction can be the consequence of either a higher or lower mtDNA-content. A change in relative mtDNAcn may be related to the development of multiple forms of disease. To date, many studies reported increases or decreases of mtDNA-content in response to endogenous or exogenous factors. Decreased mtDNA-content has been shown in diabetes type II[61,64,81], breast cancer[65,82], low birth weight[43], multiple sclerosis[63] and in response to cigarette smoke[83]. Other authors report increases in response to cigarette smoking[84], PM exposure in steel workers[59], calorie restriction and hypoxia[85]. These observations on mtDNA-content are sometimes contradictory but we must bear in mind that mtDNA-content fluctuates during aging, on the effect of different environmental factors and the tissue investigated[58,62].

4.3 Comparison of placental tissue and cord blood mtDNA

In recent studies, qPCR is widely used to quantify the mtDNA in relation to the nDNA[43,59,61,63,65,81-86]. Peripheral white blood cells have been studied for mtDNAcn in many situations. Blood is preferably used due to the relatively convenience of sample collection, less ethical concerns and sample sufficiency in comparison to tissue. However, some limitations warrant consideration. Blood runs through the entire body and may not be representative for the tissue investigated. For example, Gemma et al. postulated that umbilical cord blood is not representative for foetal tissue[43]. Also, they found no association between maternal leukocyte mtDNA-content and umbilical cord mtDNA-content, indicating that leukocyte mtDNA of cord blood may not be a good indicator of mtDNA in maternal tissue or that other *in utero* factors influence mtDNA-content of cord blood. In our study, we used tissue cells for placental measurements and leukocytes from buffy coats for cord blood measurements. We found that placental tissue had higher C_T-values than cord blood, suggesting that placental tissue yielded less relative mtDNAcn in mitochondria. This finding may merely reflect that there is a difference in investigated cell type (i) or due to the protective function of the placenta (ii).

(i) Some authors have determined the error attributable to platelets of mtDNA in human blood cells[86,87]. Platelets lack nDNA but contain numerous mitochondria. Intra-individually values range from 150 000 to 350 000 platelets per µl blood. Banas et al. showed that it was not possible to receive platelet-free cells using EDTA blood collecting tubes, even if several washing steps were appended[86]. Hence, it was readily possible that, while removing the buffy coat, amounts of blood with platelets were taken along. This platelet contamination increased the mtDNA without contributing to nDNA and affects the mtDNA/nDNA ratio. Several solutions have been proposed including platelet removal using cell sorting or magnetic sorting to enable a correct quantification in isolated cells[87]. Also, using serum tubes that contain small plastic particles, it is possible to activate and aggregate platelets to provide platelet-free cell fractions[86].

(ii) Foreign compounds can both be blocked or facilitated into the foetal compartments by placental transporters[38,88]. Compounds that do not traverse the placenta will not contribute to effects in the cord blood and thus solely on placental cells. The endocrine organ consists of different placental cells that can metabolise these xenobiotics and make them less harmful. PM_{10} compounds may escape metabolism, affecting placental mitochondrial function. This has an influence on active nutrient transfer and vascular development, impacting foetal development.

Both in placental and cord blood cells, outliers were present. The difference in copy number of the two mitochondrial genes deviated seriously compared to the rest of the population. Interestingly, all outliers had a Moroccan nationality. mtDNA from North-Africans differs from European mtDNA, due to historical genetic differences.[89]

4.4 Effects of trimester-specific exposure

Another intriguing phenomenon observed by us was that PM_{10} exposure during the third trimester of pregnancy was most closely associated with a lower mtDNA-content. This indicates a potential window for susceptibility by trimester exposure that may adversely affect mitochondrial function and foetal outcome. For example, for low birth weight and preterm birth, first and third trimester air pollution exposures have been implicated as having the most relevance[90]. In keeping with literature, Morello-Frosch et al. showed a decrease in birth weight of 7.7 g for each 10 $\mu g/cm^3$ increase in PM_{10} in the third trimester[42]. However, because a lower relative mtDNAcn was also seen during whole pregnancy, we can presume that exposure during the whole pregnancy plays an important role in the mtDNA-content.

In order to further explore the critical periods of PM_{10} by trimester exposure, we investigated how mtDNA-content was related to the first and second trimester. We saw a higher mtDNA-content during the first trimester (marginally significant) and no change during the second trimester. We observed that mtDNA-content first increased and ultimately decreased as pregnancy progressed. A possible explanation for our findings may be related to the mechanisms described earlier in Figure 12. Morphological changes in the placenta include a compromised delivery of maternal blood to the placenta or changes in foetal capillary surface areas. Indeed, PM had been shown to produce significant vasoconstriction resulting in hypoxia and nutrient deprivation[37,38]. Additionally, there may have been effects of PM exposure on PGC-1α, an essential coactivator in the expression of PPARγ, resulting in an altered trophoblast differentiation and placental development. In Adibi et al. it has already been shown that PPARγ expression was altered after phthalate exposure[88]. Given the central importance of PGC-1α in mitochondrial biogenesis and function, it is readily possible that PM exposure exert adverse effects on placental mitochondria along this pathway. A maladaptation to metabolic changes takes place, due to mutations and hypoxia. As a metabolic compensatory response, critical nuclear and mitochondrial pathways are activated for mitochondrial biogenesis to increases the mitochondrial abundance and mtDNAcn[85]. We hypothesized that these events occur as a response to PM_{10} exposure during the first trimester of pregnancy.

However, in time, as PM and PM constituents also contribute to mutations in the mtDNA, the bio-energetic and replicative function of defective mitochondria declines and results in a decrease of relative mtDNAcn and thus mtDNA content[61]. This was seen in the third trimester. What we also observed was that newborns, born in the cold season had a lower mtDNA-content than newborns born in the warm season. Generally, we can assume that newborns, born in the cold season, were exposed to higher levels of PM_{10} during the last periods of pregnancy because air pollution levels are higher in the cold season. This observation is related to the effects on the mtDNA-content.

Another possibility for the different findings during trimester exposure may be that placental structure changes during pregnancy. Complex vascular alterations are considered to be the main cause of placenta abnormalities in the second and third trimester[41]. Foreign compounds may interfere differently with placental development during different gestational periods. During the first trimester and term, the placenta expresses several CYP450 enzymes, although only a few of them are active. This indicates that metabolism of PM may be reduced and mutations occur more frequent in the first trimester. Throughout late pregnancy, a population of progenitor trophoblasts persist and undergoes renewal and differentiation. It may be that this late-stage process was disrupted by the combined effects of mutations and PM_{10} exposure, resulting in a lower mtDNA-content and mitochondrial dysfunction in the third trimester. Taken together, these proposed pathways may explain the changes of mtDNA-content to PM_{10} exposure during the three trimesters.

Yet, the question that remains to be answered is whether a reduced mitochondrial function is due to a change in mtDNA-content or abundance of mitochondria. According to Bouhours-Nouet et al. a lower relative mtDNAcn per mitochondrion is seen in placental cells after smoking exposure, rather than a quantitative decrease in the number of mitochondria[83].

4.5 Marker of traffic-related air pollution

The exposure to traffic-related air pollution is difficult to study. We measured the exposure to PM_{10} to link traffic-related air pollution and molecular effects. Distance to major road is a surrogate for traffic-related air pollution[68]. Our findings verified that PM_{10} exposure and distance to major roads are closely related. Moreover, after adjusting for confounding variables, we found a significant association between distance to major road and placental mtDNA-content. As distance to a major road increased, more mtDNAcn were present mitochondria. Each doubling in distance was associated with a significant increase of mtDNA/nDNA ratio by 5.8 % (95 % CI: 0.2 % to 6.5 %, $p = 0.0447$). This observation verifies that distance to major roads is a surrogate for traffic-related air pollution and is associated with placental mtDNA-content.

4.6 Other observational studies

Few studies have examined the relation between air pollution exposure during pregnancy and other molecular markers. To the best of our knowledge, this is the first study reporting the effects of PM_{10} exposure on placental mtDNA-content. Our findings are in agreement with an observation of Bouhours-Nouet et al. on the association between maternal smoking during pregnancy and a lower mtDNA-content in placental tissue[83]. Maternal smoking modulates mtDNA replication in a negative manner. The chemical composition of tobacco smoke is similar to fossil fuel combustion products

that contribute to air pollution. Moreover, it has been shown that environmental tobacco smoke creates high level indoor particulate pollution, with concentrations of PM_{10} exceeding air quality standards[91]. More than 3800 chemicals present in tobacco smoke may cause oxidative stress directly, via biotransformation, or by macrophage activation. Tobacco smoke also produces vascular endothelial damage.[92] These events lead to alterations in mtDNA-content as described in Figure 12. Environmental tobacco smoke presents health risks similar to those of air pollution in regard to respiratory and cardiovascular disease. In our case however, we did not observe a significant association between smoking during pregnancy and a lower mtDNA-content ($p = 0.23$) in the whole study population. When we took only Europeans into account, we saw that smoking during pregnancy had an effect on placental mtDNA-content but this did not reached significance ($p = 0.08$). This was probably due to the small group of smokers during pregnancy compared to non-smokers. Taken together, these studies suggest the possibility of transplacental transfer of pollutants from mother to foetus.

Several other studies found a relationship between birth weight and ambient air pollution[7,39-42,44,72,93]. In our case, we found neither a significant association between birth weight and PM_{10} exposure nor an association between birth weight and placental and cord blood mtDNA-content. A potential explanation might be that we measured PM_{10} exposure and most studies found an association between $PM_{2.5}$ and low birth weight[40-42,90]. Gehring et al. emphasized the importance of the sample size in these epidemiologic studies. Most studies with small sample sizes did not find an association between air pollution and birth weight. Our results may be due to the relatively small sample size.

4.7 Limitations

Contradictory results in epidemiological studies are due to many factors including differences in confounding factors, pollution measurement techniques, composition of pollutants, study sampling and statistical modelling techniques. Therefore, several limitations of the present study warrant consideration. Because personal PM exposure is relatively difficult to study, we used ambient interpolated PM values of monitoring stations to estimate period-specific exposures. To derive trimester-specific, last month of pregnancy and whole pregnancy exposures, we averaged daily exposures. For this reason, our analysis does not account for transient spikes in air pollutant levels during these terms. Also, ambient exposure does not account for indoor exposure, although we obtained information on indoor smoking. We did not obtain information on other important gaseous air pollutants including SO_2, NO_2 and CO that are associated with traffic air pollution. However, PM is a good surrogate for other associated air pollution components because it was significantly correlated with SO_2 ($r = 0.75$), NO_x ($r=0.37$) and PAHs ($r=0.79$), all $p < 0.0001$.[40] These measurement

errors could have an impact on our estimated effect of PM_{10} exposure on mtDNA-content. We tried to assess PM_{10} exposure on individually level. Nevertheless, the changes we observed in mtDNA-content do not prove causality.

Furthermore, the measurement of mtDNA-content in cord blood may have been misrepresented due to the high amount of platelets processed in DNA extraction. This led to higher mtDNAcn without contributing to nDNAcn. This may be a possible explanation why the mtDNA-content was not the same as in placental tissue.

Finally, our observations did not confirm an effect of PM exposure on the birth weight of newborns. The sample size of the present population was rather small and we encourage to verify our findings in larger populations.

4.8 Future research

It is necessary to further investigate the relation between PM_{10} exposure and mitochondrial function. First, we need to clarify the difference between mtDNA-content of placental tissue and cord blood cells. A possible solution is the usage of serum tubes to collect umbilical cord blood. In contrast to EDTA as anticoagulant, these blood collecting tubes provide platelet-free cell fractions that do not contaminate mtDNA-content with platelet mtDNA.

Second, metal-analysis of cord and maternal blood will reveal toxic metals to which pregnant mothers were exposed. The difference between the maternal and foetal circulation shows which toxic metals are blocked by the placenta and contribute to mitochondrial effects. In this study, we assumed that Cd, Ni, As, Pb or Cr separately or combined, made part of PM_{10} fraction.

Third, it is useful to measure the expression of PGC-1α, an essential coactivator that interacts with nuclear receptors and transcription factors that activate genes responsible for mitochondrial biogenesis and function. Combining mtDNA-content observations with expression of this coactivator provides more insight into possible mechanisms linking PM exposure and mitochondrial dysfunction.

Fourth, we could also measure insulin-like growth factors in cord blood. Insulin-like growth factors are synthesized in the placenta and are essential in foetal growth. Investigating these factors may give us more insight into foetal health outcomes because the lack of significance of birth weight was probably due to the small sample size.

Fifth, by determining polymorphisms in biotransformation genes (e.g. CYP1A1), we are able to detect genetic differences in groups of people particularly susceptible to PM_{10} exposure. For example, newborns with Moroccan origin have genetic differences compared to Europeans, as seen in mtDNA.

Sixth, DNA adducts of leukocyte DNA and placental DNA can be analysed because constituents of PM including PAHs and volatile organic compounds can form these adducts.

Seventh, we can study gene expression patterns of genes involved in inflammation including heme oxygenase-1, interleukine-6 and interleukine-8 from placental tissue and leukocytes of cord blood. In this way, we will better understand the role of inflammation on oxidative stress and placental mitochondrial function.

Finally, the determination of telomere length in placental tissue and white blood cells of cord blood could provide us another marker of effect in addition to mtDNA-content. The *in utero* telomere length is synchronized (i.e. similar) among tissues of the same fetus but highly variable during extra-uterine life[94,95]. Hoxha et al. 2009 observed that telomere shortening is associated with exposure to traffic pollution, an inducer of oxidative stress and inflammation[96]. Whether exposure to particulate matter and toxic metals is related to *in utero* telomere shortening has never been determined. Moreover, Sahin et al. 2011 recently showed a direct link between telomere dysfunction and mitochondrial biology[79]. Given the central importance of mitochondria, one might anticipate that this telomere dysfunction lies at the base of decreased mitochondrial biogenesis/activity and can be related to mitochondrial function.

5 Conclusion and perspectives

In conclusion, we showed significant alterations of mtDNA-content in placentas in response to *in utero* PM_{10} exposure during the last period of pregnancy. Each 10 µg/cm^3 increase in PM_{10} during the third trimester of pregnancy was associated with a lower placental mtDNA/nDNA ratio of $\beta = -0.161$ (95 % CI: -0.094 to -0.229; $p < 0.0001$). This indicates a potential window for susceptibility by trimester exposure that may adversely affect mitochondrial function and possibly foetal outcome. Furthermore, each doubling in distance was associated with an increase in mtDNA/nDNA ratio by 5.8 % (95 % CI: 0.2 % to 6.5 %, $p = 0.04$). This observation verifies that distance to roads is a surrogate for traffic-related air pollution and is associated with placental mtDNA-content[68]. In contrast, no changes were seen in the mtDNA-content of cord blood.

The implication of mtDNA-content alterations in adverse foetal health outcomes remains to be determined. The mechanisms controlling these processes are not yet fully elucidated but our findings deliver substantial evidence that environmental exposures play a role. This is of importance for PM regulation to protect the more susceptible segment of the population. We encourage to broaden our perspective in a larger study population to link PM_{10} exposure, but also toxic metals, to mitochondrial function of placental tissue and the resulting adverse foetal health outcomes including low birth weight.

References

1. Nriagu JO. A History of Global Metal Pollution. *Science* 1996;**272**(5259):223.
2. World Health Organisation. Health Aspects of Air Pollution with Particulate Matter, Ozone and Nitrogen Dioxide. 2003:7-9.
3. Nawrot TS, Adcock I. The Detrimental Health Effects of Traffic-related Air Pollution: A Role for DNA Methylation? *Am J Respir Crit Care Med* 2009;**179**(7):523-524.
4. Li N, Xian T, Nel AE. The Role of Oxidative Stress in Ambient Particulate Matter-induced Lung Diseases and Its Implications in the Toxicity of Engineered Nanoparticles. *Free Radic Biol Med* 2008;**44**(9):1689–1699.
5. Maitre A, Bonneterre V, Huillard L, Sabatier P, de Gaudemaris R. Impact of urban atmospheric pollution on coronary disease. *Eur Heart J* 2006;**27**(19):2275-2284.
6. Nawrot TS, Torfs R, Fierens F, De Henauw S, Hoet PH, Van Kersschaever G, De Backer G, Nemery B. Stronger associations between daily mortality and fine particulate air pollution in summer than in winter: evidence from a heavily polluted region in western Europe. *Journal of Epidemiology and Community Health* 2007;**61**(2):146-149.
7. Pereira LA, Loomis D, Conceicao GM, Braga ALF, Arcas RM, Kishi HS, Singer JM, Bohm GM*, et al.* Association between air pollution and intrauterine mortality in Sao Paulo, Brazil. *Environmental Health Perspectives* 1998;**106**:325 - 329.
8. Peters A, Frohlich M, Doring A, Immervoll T, Wichmann HE, Hutchinson WL, Pepys MB, Koenig W. Particulate air pollution is associated with an acute phase response in men; results from the MONICA-Augsburg Study. *Eur Heart J* 2001;**22**(14):1198-1204.
9. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate Stephen T, Frew A. Acute Inflammatory Responses in the Airways and Peripheral Blood After Short-Term Exposure to Diesel Exhaust in Healthy Human Volunteers. *Am J Respir Crit Care Med* 1999;**159**(3):702-709.
10. Moller P, Folkmann JK, Forchhammer L, Brauner EV, Danielsen PH, Risom L, Loft S. Air pollution, oxidative damage to DNA, and carcinogenesis. *Cancer Lett* 2008;**266**(1):84-97.
11. Deutsch F, Schrooten L, Broeckx S, Vankerkom J. Milieurapport Vlaanderen, Achtergronddocument, Verspreiding van zwevend stof. 2007:7-14.
12. Weijers EP, Even A, Kos GPA, Groot ATJ, Erisman JW, ten Brink HM. Particulate matter in urban air: health risks, intrumentation and measurements, and political awareness. *Urban air quality* 2001.
13. Brauer M, Avila-Casado C, Fortoul TI, Vedal S, Stevens B, Churg A. Air pollution and retained particles in the lung. *Environ Health Perspect* 2001;**109**(10):1039-1043.
14. Rossner P, Jr., Tabashidze N, Dostal M, Novakova Z, Chvatalova I, Spatova M, Sram RJ. Genetic, biochemical, and environmental factors associated with pregnancy outcomes in newborns from the Czech Republic. *Environ Health Perspect* 2011;**119**(2):265-271.
15. Rijks- M-eNedsMeVvh, Milieu ivVe. Fijn stof nader bekeken: De stand van zaken in het dossier fijn stof. 2005:12-14.
16. den Hartigh LJ, Lamé MW, Ham W, Kleeman MJ, Tablin F, Wilson DW. Endotoxin and polycyclic aromatic hydrocarbons in ambient fine particulate matter from Fresno, California initiate human monocyte inflammatory responses mediated by reactive oxygen species. *Toxicology in Vitro* 2010;**24**(7):1993-2002.
17. Nemmar A, Hoet PHM, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L*, et al.* Passage of Inhaled Particles Into the Blood Circulation in Humans. *Circulation* 2002;**105**(4):411-414.
18. Churg A, Brauer M. Human lung parenchyma retains PM2.5. *Am J Respir Crit Care Med* 1997;**155**(6):2109-2111.

19. Maes J BJ, Provoost J, Den Hond E. Milieurapport Vlaanderen, Achtergronddocument, Verspreiding van zware metalen. 2007:11-13, 31.
20. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Cadmium and cadmium compounds (accessed January 3, 2011). 1993.
21. Van Sittert NJ, Ribbens PH, Huisman B, Lugtenburg D. A nine year follow up study of renal effects in workers exposed to cadmium in a zinc ore refinery. *Br J Ind Med* 1993;**50**(7):603-612.
22. World Health Organisation. Air quality guidelines for Europe. WHO Reg Publ Eur Ser. 2001/05/25 ed, 2000.
23. Nawrot TS, Staessen JA, Roels HA, Munters E, Cuypers A, Richart T, Ruttens A, Smeets K*, et al.* Cadmium exposure in the population: from health risks to strategies of prevention. *Biometals* 2010;**23**(5):769-782.
24. Nordberg G, Nogawa K, Nordberg M, Friberg L. Cadmium. In: Nordberg GF, Fowler BF, Norberg M et al. Handbook on the toxicology of metals. Third edition ed. Amsterdam: *Elsevier*, 2007.
25. Nawrot TS, Van Hecke E, Thijs L, Richart T, Kuznetsova T, Jin Y, Vangronsveld J, Roels HA*, et al.* Cadmium-related mortality and long-term secular trends in the cadmium body burden of an environmentally exposed population. *Environ Health Perspect* 2008;**116**(12):1620-1628.
26. Nawrot T, Geusens P, Nulens TS, Nemery B. Occupational cadmium exposure and calcium excretion, bone density, and osteoporosis in men. *Journal of Bone and Mineral Research* 2010;**25**(6):1441-1445.
27. Nawrot T, Plusquin M, Hogervorst J, Roels HA, Celis H, Thijs L, Vangronsveld J, Van Hecke E*, et al.* Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *The Lancet Oncology* 2006;**7**(2):119-126.
28. Sharma VK, Sohn M. Aquatic arsenic: toxicity, speciation, transformations, and remediation. *Environ Int* 2009;**35**(4):743-759.
29. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Chromium, Nickel and Welding. *IARC Monographs* 1997;**49**.
30. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Arsenic in drinking-water (accessed January 3, 2011). 2004.
31. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Inorganic and Organic Lead Compounds (accessed on January 3, 2011). 2006.
32. Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro. *Thorax* 1996;**51**(12):1216-1222.
33. Donaldson K, Stone V. Current hypotheses on the mechanisms of toxicity of ultrafine particles. *Ann Ist Super Sanita* 2003;**39**(3):405-410.
34. Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environ Health Perspect* 2001;**109 Suppl 4**:523-527.
35. Nawrot TS, Nemmar A, Nemery B. Air pollution: to the heart of the matter. *European Heart Journal* 2006;**27**(19):2269-2271.
36. Sram RJ, Binkova B, Dejmek J, Bobak M. Ambient air pollution and pregnancy outcomes: a review of the literature. *Environmental Health Perspectives* 2005;**113**:375 - 382.
37. Myllynen P, Pasanen M, Pelkonen O. Human placenta: a human organ for developmental toxicology research and biomonitoring. *Placenta* 2005;**26**(5):361-371.
38. Veras MM, Damaceno-Rodrigues NR, Caldini EG, Maciel Ribeiro AA, Mayhew TM, Saldiva PH, Dolhnikoff M. Particulate urban air pollution affects the functional morphology of mouse placenta. *Biol Reprod* 2008;**79**(3):578-584.
39. Wang X, Ding H, Ryan L, Xu X. Association between air pollution and low birth weight: a community based study. *Environmental Health Perspectives* 1997;**105**:514 - 520.
40. Dejmek J, Selevan SG, Benes I, Solansky I, Sram RJ. Fetal growth and maternal exposure to particulate matter during pregnancy. *Environ Health Perspect* 1999;**107**(6):475-480.

41. Jalaludin B, Mannes T, Morgan G, Lincoln D, Sheppeard V, Corbett S. Impact of ambient air pollution on gestational age is modified by season in Sydney, Australia. *Environmental Health* 2007;**6**(1):16.
42. Morello-Frosch R, Jesdale BM, Sadd JL, Pastor M. Ambient air pollution exposure and full-term birth weight in California. *Environ Health* 2010;**9**:44.
43. Gemma C, Sookoian S, Alvarinas J, Garcia SI, Quintana L, Kanevsky D, Gonzalez CD, Pirola CJ. Mitochondrial DNA Depletion in Small- and Large-for-Gestational-Age Newborns[ast]. *Obesity* 2006;**14**(12):2193-2199.
44. Bobak M. Outdoor air pollution, low birth weight, and prematurity. *Environmental Health Perspectives* 2000;**108**:173 - 176.
45. Ritz B, Yu F, Chapa G, Fruin S. Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. *Epidemiology* 2000;**11**:502 - 511.
46. Falcón M, Viñas P, Luna A. Placental lead and outcome of pregnancy. *Toxicology* 2003;**185**(1-2):59-66.
47. Salpietro CD, Gangemi S, Minciullo PL, Briuglia S, Merlino MV, Stelitano A, Cristani M, Trombetta D*, et al.* Cadmium concentration in maternal and cord blood and infant birth weight: a study on healthy non-smoking women. *Journal of Perinatal Medicine* 2002;**30**(5):395-399.
48. Barker DJ. Fetal origins of coronary heart disease. *BMJ* 1995;**311**(6998):171-174.
49. Barker DJP, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *The Lancet* 1986;**327**(8489):1077-1081.
50. Made KR, Diederick EG, Michiel LB, Manuel Castro C, Lydia EV, Anath O, Cuno SPMU. Lower birth weight predicts metabolic syndrome in young adults: The Atherosclerosis Risk in Young Adults (ARYA)-study. *Atherosclerosis* 2006;**184**(1):21-27.
51. Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *BMJ* 1991;**303**(6804):671-675.
52. Cooper C, Javaid K, Westlake S, Harvey N, Dennison E. Developmental Origins of Osteoporotic Fracture: the Role of Maternal Vitamin D Insufficiency. *The Journal of Nutrition* 2005;**135**(11):2728S-2734S.
53. Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol* 2006;**141**(2):312-322.
54. Risom L, Moller P, Loft S. Oxidative stress-induced DNA damage by particulate air pollution. *Mutat Res* 2005;**592**(1-2):119-137.
55. Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T*, et al.* Ultrafine Particulate Pollutants Induce Oxidative Stress and Mitochondrial Damage. *Environ Health Perspect* 2003;**111**(4).
56. Mehta M, Chen LC, Gordon T, Rom W, Tang MS. Particulate matter inhibits DNA repair and enhances mutagenesis. *Mutat Res* 2008;**657**(2):116-121.
57. Taanman JW. The mitochondrial genome: structure, transcription, translation and replication. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1999;**1410**(2):103-123.
58. Clay Montier LL, Deng JJ, Bai Y. Number matters: control of mammalian mitochondrial DNA copy number. *Journal of Genetics and Genomics* 2009;**36**(3):125-131.
59. Hou L, Zhu Z-Z, Zhang X, Nordio F, Bonzini M, Schwartz J, Hoxha M, Dioni L*, et al.* Airborne particulate matter and mitochondrial damage: a cross-sectional study. *Environmental Health* 2010;**9**(1):48.
60. Lamson DW, Plaza SM. Mitochondrial factors in the pathogenesis of diabetes: a hypothesis for treatment. *Altern Med Rev* 2002;**7**(2):94-111.
61. Wong J, McLennan SV, Molyneaux L, Min D, Twigg SM, Yue DK. Mitochondrial DNA content in peripheral blood monocytes: relationship with age of diabetes onsetand diabetic complications. *Diabetologia* 2009;**52**(9):1953-1961.

62. Andreu AL, Martinez R, Marti R, Garcia-Arumi E. Quantification of mitochondrial DNA copy number: pre-analytical factors. *Mitochondrion* 2009;**9**(4):242-246.
63. Blokhin A, Vyshkina T, Komoly S, Kalman B. Variations in Mitochondrial DNA Copy Numbers in MS Brains. *Journal of Molecular Neuroscience* 2008;**35**(3):283-287.
64. Choi YS, Kim S, Pak YK. Mitochondrial transcription factor A (mtTFA) and diabetes. *Diabetes Research and Clinical Practice* 2001;**54**(Supplement 2):S3-S9.
65. Yu M, Zhou Y, Shi Y, Ning L, Yang Y, Wei X, Zhang N, Hao X*, et al.* Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. *IUBMB Life* 2007;**59**(7):450-457.
66. Glinianaia SV, Rankin J, Bell R, Pless-Mulloli T, Howel D. Particulate air pollution and fetal health: a systematic review of the epidemiologic evidence. *Epidemiology* 2004;**15**(1):36-45.
67. Adibi JJ, Hauser R, Williams PL, Whyatt RM, Thaker HM, Nelson H, Herrick R, Bhat HK. Placental biomarkers of phthalate effects on mRNA transcription: application in epidemiologic research. *Environ Health* 2009;**8**:20.
68. Hoek G, Brunekreef B, Goldbohm S, Fischer P, van den Brandt PA. Association between mortality and indicators of traffic-related air pollution in the Netherlands: a cohort study. *Lancet* 2002;**360**(9341):1203-1209.
69. Applied Biosystems. MagMAX^{TM} Express 96 User Manual. 2008;16-18.
70. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007;**8**(2):R19.
71. www.zorg-en-gezondheid.be/cijfers/geboorte-en-bevolking/geboortegewicht-van-eenlingen/ (based on data from the Study centre for Perinatal Epidemiology in Flanders).
72. Ballester F, Estarlich M, Iniguez C, Llop S, Ramon R, Esplugues A, Lacasana M, Rebagliato M. Air pollution exposure during pregnancy and reduced birth size: a prospective birth cohort study in Valencia, Spain. *Environ Health* 2010;**9**:6.
73. Kannan S, Misra DP, Dvonch JT, Krishnakumar A. Exposures to airborne particulate matter and adverse perinatal outcomes: a biologically plausible mechanistic framework for exploring potential effect modification by nutrition. *Environ Health Perspect* 2006;**114**(11):1636-1642.
74. Watson AL, Skepper JN, Jauniaux E, Burton GJ. Susceptibility of Human Placental Syncytiotrophoblastic Mitochondria to Oxygen-Mediated Damage in Relation to Gestational Age. *Journal of Clinical Endocrinology & Metabolism* 1998;**83**(5):1697-1705.
75. Li Z, Hyseni X, Carter JD, Soukup JM, Dailey LA, Huang YC. Pollutant particles enhanced H2O2 production from NAD(P)H oxidase and mitochondria in human pulmonary artery endothelial cells. *Am J Physiol Cell Physiol* 2006;**291**(2):C357-365.
76. Huang YC, Ghio AJ. Vascular effects of ambient pollutant particles and metals. *Curr Vasc Pharmacol* 2006;**4**(3):199-203.
77. Peretz A, Sullivan JH, Leotta DF, Trenga CA, Sands FN, Allen J, Carlsten C, Wilkinson CW*, et al.* Diesel exhaust inhalation elicits acute vasoconstriction in vivo. *Environ Health Perspect* 2008;**116**(7):937-942.
78. Miglio G, Rosa AC, Rattazzi L, Collino M, Lombardi G, Fantozzi R. PPARgamma stimulation promotes mitochondrial biogenesis and prevents glucose deprivation-induced neuronal cell loss. *Neurochem Int* 2009;**55**(7):496-504.
79. Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, Cooper M, Kotton D*, et al.* Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature* 2011;**470**(7334):359-365.
80. Dejmek J, Solansky I, Benes I, Lenicek J, Sram RJ. The impact of polycyclic aromatic hydrocarbons and fine particles on pregnancy outcome. *Environ Health Perspect* 2000;**108**(12):1159-1164.
81. Gianotti TF, Sookoian S, Dieuzeide G, Garcia SI, Gemma C, Gonzalez CD, Pirola CJ. A decreased mitochondrial DNA content is related to insulin resistance in adolescents. *Obesity (Silver Spring)* 2008;**16**(7):1591-1595.

82. Xia P, An HX, Dang CX, Radpour R, Kohler C, Fokas E, Engenhart-Cabillic R, Holzgreve W*, et al.* Decreased mitochondrial DNA content in blood samples of patients with stage I breast cancer. *BMC Cancer* 2009;**9**:454.
83. Bouhours-Nouet N, May-Panloup P, Coutant R, de Casson FB, Descamps P, Douay O, Reynier P, Ritz P*, et al.* Maternal smoking is associated with mitochondrial DNA depletion and respiratory chain complex III deficiency in placenta. *Am J Physiol Endocrinol Metab* 2005;**288**(1):E171-177.
84. Masayesva BG, Mambo E, Taylor RJ, Goloubeva OG, Zhou S, Cohen Y, Minhas K, Koch W*, et al.* Mitochondrial DNA content increase in response to cigarette smoking. *Cancer Epidemiol Biomarkers Prev* 2006;**15**(1):19-24.
85. Lattuada D, Colleoni F, Martinelli A, Garretto A, Magni R, Radaelli T, Cetin I. Higher mitochondrial DNA content in human IUGR placenta. *Placenta* 2008;**29**(12):1029-1033.
86. Banas B, Kost BP, Goebel FD. Platelets, a typical source of error in real-time PCR quantification of mitochondrial DNA content in human peripheral blood cells. *Eur J Med Res* 2004;**9**(8):371-377.
87. Cossarizza A, Riva A, Pinti M, Ammannato S, Fedeli P, Mussini C, Esposito R, Galli M. Increased mitochondrial DNA content in peripheral blood lymphocytes from HIV-infected patients with lipodystrophy. *Antivir Ther* 2003;**8**(4):315-321.
88. Adibi JJ, Whyatt RM, Hauser R, Bhat HK, Davis BJ, Calafat AM, Hoepner LA, Perera FP*, et al.* Transcriptional biomarkers of steroidogenesis and trophoblast differentiation in the placenta in relation to prenatal phthalate exposure. *Environ Health Perspect* 2010;**118**(2):291-296.
89. Ennafaa H, Cabrera VM, Abu-Amero KK, Gonzalez AM, Amor MB, Bouhaha R, Dzimiri N, Elgaaied AB*, et al.* Mitochondrial DNA haplogroup H structure in North Africa. *BMC Genet* 2009;**10**:8.
90. Gehring U, Wijga AH, Fischer P, de Jongste JC, Kerkhof M, Koppelman GH, Smit HA, Brunekreef B. Traffic-related air pollution, preterm birth and term birth weight in the PIAMA birth cohort study. *Environ Res*;**111**(1):125-135.
91. Invernizzi G, Ruprecht A, Mazza R, Rossetti E, Sasco A, Nardini S, Boffi R. Particulate matter from tobacco versus diesel car exhaust: an educational perspective. *Tob Control* 2004;**13**(3):219-221.
92. Weber C, Erl W, Weber K, Weber PC. Increased adhesiveness of isolated monocytes to endothelium is prevented by vitamin C intake in smokers. *Circulation* 1996;**93**(8):1488-1492.
93. Maisonet M, Bush TJ, Correa A, Jaakkola JJK. Relation between ambient air pollution and low birth weight in the Northeastern United States. *Environmental Health Perspectives* 2001;**109**:351 - 356.
94. Friedrich U, Schwab M, Griese EU, Fritz P, Klotz U. Telomeres in neonates: new insights in fetal hematopoiesis. *Pediatr Res* 2001;**49**(2):252-256.
95. Okuda K, Bardeguez A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, Skurnick J, Awad G*, et al.* Telomere length in the newborn. *Pediatr Res* 2002;**52**(3):377-381.
96. Hoxha M, Dioni L, Bonzini M, Pesatori AC, Fustinoni S, Cavallo D, Carugno M, Albetti B*, et al.* Association between leukocyte telomere shortening and exposure to traffic pollution: a cross-sectional study on traffic officers and indoor office workers. *Environ Health* 2009;**8**:41.

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