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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Cellular DNA damage-related genes in association with *in utero* parti culate matter exposure: p53 as a central hub

Promotor :
Prof. dr. Tim NAWROT

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dr. Karen VRIJENS

Livia Vanderheijden
Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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List of Abbreviations

ACTB	Actin, beta
B2M	Beta-2-microglobulin
BAX	BCL2-associated X protein
cDNA	Complementary DNA
CYC1	Cytochrome c-1
DNMT1	DNA (cytosine-5-)-methyltransferase 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IPO8	Importin 8
MN	Micronuclei
p21	Cyclin-dependent kinase inhibitor 1A
p53	Tumour suppressor protein *p53*
PAH	Polycyclic aromatic hydrocarbons
PCNA	Proliferating cell nuclear antigen
PM	Particulate matter
PM_{10}	Particulate matter with an aerodynamic diameter of ≤ 10 µm
$PM_{2.5}$	Particulate matter with an aerodynamic diameter of ≤ 2.5 µm
$PM_{0.1}$	Particulate matter with an aerodynamic diameter of ≤ 0.1 µm
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A, 220kDa
qPCR	Quantitive real-time polymerase chain reaction
ROS	Reactive oxygen species
RPLP0	Ribosomal protein, large, P0
TBP	TATA box binding protein
UBC	Ubiquitin C
WHO	World Health Organization
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

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Abstract

Background: Micronuclei (MN) are small nuclei, which are not incorporated in the daughter nuclei during cell division. They are formed as a result of chromosomal aberrations, and hence a valid biomarker for genotoxicity. DNA adducts and MN frequencies have been associated with *in utero* air pollution exposure in cord blood. Van Leeuwen et al. published a MN formation network with *p53* as a central hub. The *p53* gene encodes for the tumour suppressor protein which regulates incoming stress signals and has an important role in many biological pathways.

Hypothesis: We hypothesized that expression of genes involved in the MN formation network is altered in association with *in utero* $PM_{2.5}$ exposure in newborns.

Methods: Cord blood was retrieved from 96 newborns and gene expression of *p53*, *DNMT1*, *PCNA*, *BAX* and *p21* was determined by quantitative real-time polymerase chain reaction.

Results: The key finding of this study was the positive association between *in utero* $PM_{2.5}$ exposure during the first trimester of pregnancy and gene expression of *p53* (3.21%, 95% CI: 0.41 to 0.61, p=0.03 for each 5 µg/m^3 increase in $PM_{2.5}$). In addition, expression of *p53* was significantly associated with *DNMT1* (r=0.26, p=0.01) and modestly associated with *BAX* (r=0.19, p=0.06) and *PCNA* (r=0.18, p=0.08). No significant association was found with gene expression of *p21*. Furthermore, we observed no direct significant association between $PM_{2.5}$ exposure and the genes in the network individually, neither between $PM_{2.5}$ exposure and the entire MN formation network, however a positive trend was observed.

Conclusion: Our results suggest that $PM_{2.5}$ exposure affects *p53* gene expression in cord blood. In this way, *p53* acts as a central hub for the regulation of MN formation or other responsive mechanisms associated with cellular damage that we have not investigated. Our findings are a first step into transcriptomic analyses of MN-related genes in response to environmental stress. Research on genes involved in MN formation and exposure to chemicals will make it possible to eventually develop a reliable reporter gene assay to screen a chemically exposed human population in a fast and less labour intensive manner.

Samenvatting

Achtergrond: Micronuclei zijn kleine kernen die niet opgenomen zijn in de dochterkern. Deze kernen zijn ontstaan als gevolg van chromosomale breuken, en zijn dus een gevalideerde marker voor genotoxische schade. Micronuclei frequentie is al geassocieerd met blootstelling aan genotoxische componenten en omgevingsfactoren zoals luchtvervuiling en met DNA-schade in navelstrengbloed. Van Leeuwen et al. hebben een MN-formatie netwerk gepubliceerd met *p53* als centraal gen. *P53* is een tumor suppressor gen dat een centrale rol speelt in verschillende biologische paden.

Hypothese: Onze hypothese stelt dat genexpressie van genen betrokken in het micronuclei-formatie netwerk verandert in associatie met *in utero* blootstelling aan fijn stof bij pasgeborenen.

Methode: Navelstrengbloed van 96 pasgeborenen werd verzameld en genexpressie van *p53*, *BAX*, *PCNA*, *p21* en *DNMT1* werd gemeten met qPCR.

Resultaten: De belangrijkste bevinding van deze studie was de positieve associatie tussen in *utero* blootstelling aan $PM_{2.5}$ tijdens het eerst trimester van de zwangerschap en de genexpressie van *p53.* (3.21%, 95% BI:0.41 tot 0.61, p=0.03 voor elke 5 µg/m^3 verhoging in $PM_{2.5}$). Expressie van *p53* was ook significant geassocieerd met expressie van *DNMT1* (r=0.26, p=0.01) en modest geassocieerd met genexpressie van *BAX* (r=0.19, p=0.06) en *PCNA* (r=0.18, p=0.08). Geen significante associatie werd gevonden met genexpressie van *p21*. Verder hebben we geen direct significant verband geobserveerd tussen $PM_{2.5}$ blootstelling en de genen in het netwerk afzonderlijk, noch tussen $PM_{2.5}$ blootstelling en het gehele micronuclei-formatie netwerk. Er werd wel een positieve trend waargenomen.

Conclusie: Onze resultaten suggereren dat *in utero* $PM_{2.5}$ blootstelling tijdens de zwangerschap de *p53* genexpressie in navelstrengbloed beïnvloedt. Op deze manier fungeert *p53* als een centraal knooppunt voor de regulering van micronuclei vorming of andere responsieve mechanismen van cellulaire schade die we niet hebben onderzocht. Onze bevindingen zijn een eerste stap in analyse op transcriptie-niveau van micronuclei-gerelateerde genen in reactie op omgevingsfactoren en stress.

1. Introduction

1.1 Environmental air pollution

The growing industry and increasing traffic density have lead to the formation of pollutants that do not originally appear in the atmosphere. Air pollutants can be emitted into the atmosphere (primary air pollutants) or formed within the atmosphere (secondary air pollutants) and can be present in the form of solid particles, liquid droplets, or gases. Over the last decade, air pollution levels have dropped significantly but the consequences for environment and human health are still present. Exposure to environmental air pollution can cause a range of effects and diseases depending on acute or chronic exposure and quantity of exposure. Many health effects are well known and can vary from cardiovascular disease, respiratory irritation and disease to lung cancer and asthmatic attacks (1-3).

Most dangerous for human health is fine dust or particulate matter (PM), which is a complex mixture that consists of microscopic particles and liquid droplets made up of acids, organic chemicals, metals and soil or dust particles (4). PM can originate from anthropogenic (e.g. vehicle emissions, tobacco smoke, industrial combustion processes) or natural sources (e.g. dust storms, volcano eruptions, fires). Diesel cars and combustion of biomass causes black carbon and other combustion-related materials which are components of PM. The World Health Organisation (WHO) classified black carbon as group 1 carcinogenic for humans (5).

PM can be subdivided by its aerodynamic equivalent diameter and the place it will deposit in human airways. Particles with a diameter between 2.5 and 10 µm are defined as "coarse" (PM_{10}), less than 2.5 µm as "fine" ($PM_{2.5}$) and less than 0.1 µm as "ultrafine" particles ($PM_{0.1}$). The size of the particles determines the effect on human health (6). Particles with a diameter exceeding 10 µm are largely filtered out by the nose and upper airway. The smaller the particles are, the deeper they deposit in the lungs; ultrafine particles can even reach the terminal alveoli and the bloodstream. It is estimated that exposure to $PM_{2.5}$ can reduce lifetime of the Belgian population with 9 to 10 months (7). Inhalation of PM causes irritation or damage to tissue in the lungs. Acute exposure to PM can aggravate pre-existing problems, like respiratory infections and asthma (8). Chronic exposure increases the risk of cardiovascular disorders and lung diseases, including lung cancer (9).

These negative consequences for human health resulted in European standards for air quality set by the European Union, which were based on the specific recommendations set by the WHO. The guidelines set by the WHO are not legally imposed, but are considered to be the acceptable level of air pollution, regarding potential impact on public health and environment, so most countries try to reach them. In 2005, the WHO updated the Air Quality Guidelines for PM, nitrogen dioxide, sulphur dioxide and ozone. These guidelines are based on a review of the evidence on the relationship between air quality and adverse human health effects. European guidelines are less strict since they do not only take into account health reasons, but also the economic feasibility to reach the guidelines. The guidelines of the WHO are only based on its goal to minimize the effects on human health (10, 11).

Table 1 gives an overview of the limits set by European Union and the WHO and the actual values in 2012 in Belgium. These European guidelines are mostly reached in Belgium, but guidelines of the WHO are often exceeded. As indicated in Table 1, the European guideline for PM_{10} is respected everywhere in

Belgium. However, the number of days on which the daily average PM_{10} concentration was higher than 50 µg/m³, is still above the permitted 35 days. Also the average concentration of $PM_{2.5}$ remained below the EU guidelines in 2012. But a comparison with WHO guidelines is less positive. Efforts have already been made to reach the guidelines, but extra effort is necessary.

Table 1: Guidelines concerning particulate matter set by Europe and the WHO.

	2012			European guidelines	WHO guidelines
	Min.	**Mean**	**Max.**		
PM_{10}					
Year average (µg/m³)	8	19	31	40	20
Number of days >50µg/m³	0	14	41	Max. 35 days	Max. 3 days
$PM_{2.5}$					
Year average (µg/m³)	6	12	21	25	10

Air pollution levels show substantial spatial and temporal variation, so exposure is different for every human being and differs according to daily activities. The effects of environmental air pollution are difficult to describe on individual level, but have a great impact on population level, particularly on susceptible groups including elderly and sick people. Another group that is more susceptible to these exposures are foetuses, newborns and children, who are very vulnerable due to their physiological immaturity. Harmful environmental factors can have a detrimental effect on their health and development. It has been shown that inhaled particles including $PM_{2.5}$ and PM_{10} can have an adverse effect on placental function by increasing blood viscosity and therefore can restrict foetal growth (12-14).

1.2 Underlying molecular mechanisms of particulate matter exposure

The negative influence of ambient air pollution on foetuses can be explained by the Barker hypothesis, which is also known as foetal origin of adult disease. This hypothesis postulates that *in utero* influences during early life can lead to permanent changes in physiology and metabolism, which can trigger the development of diseases later in life (15). Negative birth outcomes, including reduced birth size, have a well established association with an increased risk of coronary heart disease, impaired neurodevelopment, hypertension, stroke, respiratory infections and diabetes in adulthood (16, 17). Likewise, foetal and placental size have been associated with an increased risk of hypertension in adult life (18). The underlying mechanisms of adverse birth outcomes are still poorly understood, but it is assumed that these associations can be explained by nutritional stimuli, excessive hormone exposure (e.g. glucocorticoid), and also by exposure to environmental stimuli such as cigarette smoke and air pollution. Several epidemiological studies showed that malnutrition during the pregnancy leads to low birth weight, small placental size and an increased risk of coronary heart disease later in life (19-21). Associations between maternal exposure to PM and a range of adverse pregnancy outcomes, including preterm birth and low birth weight, have also been reported in several studies (22-24).

Consequently, the foetus adapts itself and permanently "programs" developmental changes that form the origin of diseases that may arise in later life. These changes include alterations in gene expression or epigenetic modifications, including alterations in DNA methylation patterns or histone modifications (15). Maternal smoking during pregnancy and differential methylation across the genome have been associated in cord blood (25).

In literature, several hypothesis are proposed that explain the specific mechanisms through which PM causes adverse foetal health effects. One hypothesis states that epithelial cells exposed to ultrafine particles can induce oxidative stress or cause inflammation by stimulating the production of the cytokine interleukine-8 (26). In addition, toxic metals, that make part of particles, also play an important role in the pro-inflammatory effects of PM_{10} (27). Transition metals catalyze the conversion of free radicals (e.g. superoxide) into more damaging compounds (e.g. hydroxyl radicals) by oxidoreduction reactions. These radicals are capable of inducing oxidative stress and damage within biological systems (28). Overall, it is assumed that disruptions, caused by an increase in oxidative stress, can affect the exchange between mother and foetus and therefore foetal development (29). It is understood that this will trigger complications within the ageing mechanisms which will lead to the development of age-related illnesses later in life.

The association between exposure to PM and its effects on ageing mechanisms in newborns, exemplified by DNA methylation and mitochondrial DNA content, has been evaluated within our research group. Previously we showed that particle exposure during early life is linked with a decrease in global DNA methylation of placental tissue (30). Recent research also observed a negative association between PM exposure and mitochondrial DNA content in placental tissue, suggesting that mitochondrial damage and inflammation can be linked to *in utero* PM exposure (31).

1.3 Cord blood as biological matrix

Bio-specimens, including urine, saliva, blood, cord blood and breast milk can be used to assess the exposure to environmental hazards of children in early childhood and foetal stage. Cord blood and placental tissue are most interesting for assessing exposure of the foetus, since collection is non-invasive for mother and child.

The placenta plays an important role in foetal growth and development, so this matrix is ideal for investigating the association between exposure to air pollution and adverse birth effects (32). However, the placenta is an extra embryonic tissue, whose genetic and epigenetic pattern may not exactly resemble this of somatic tissues. To look at the aetiology of diseases later in life, it might be more interesting to investigate somatic tissues like umbilical cord blood. Cord blood has been used in several studies investigating prenatal exposure to environmental factors (e.g. carcinogenic polycyclic aromatic hydrocarbons (PAH)) and development of diseases later in life (33-35).

1.4 Tumour suppressor gene *p53*: the central hub of cellular DNA damage

The *p53* gene encodes for the tumour suppressor protein which acts as a central hub for incoming stress signals and has an important role in many biological pathways. It can act as a sequence-specific transcription factor to regulate expression of different target genes. The exact number of *p53*-responsive genes is still unclear, but is likely to be over a hundred. Only for a small group of target genes the direct role with *p53* has been elucidated (36).

Many types of stress including DNA damage, telomere attrition, oncogene activation, loss of normal growth and survival signals and hypoxia, can activate *p53* transcription and its downstream response pathways (37). *P53* can act as a transcription factor that binds to specific sites in the regulatory regions

of *p53*-responsive genes and transcription will be activated. Depending on the severity of the DNA damage, this will result in DNA repair or apoptosis. Other possible cellular responses are differentiation of cells, senescence, inhibition of angiogenesis or growth arrest. It is established that *p53* induces apoptosis by a pathway that is dependent on ROS (reactive oxygen species) production (38).

P53 transcription can also be activated by exposure to environmental factors, including ambient air pollution, UV radiation or chemical mutagens. It has been shown in experimental research that expression of *p53* is induced by PAHs *in vitro* (39). Exposure to environmental contaminants such as PM, leads to an increased level of DNA damage, including DNA adducts, chromosome aberrations, sister chromatid exchanges, DNA strand breaks and finally *p53* expression (40, 41). Therefore, *p53* expression is accepted as a universal marker of genotoxic stress and DNA damage.

The underlying mechanisms by which *p53* modulates apoptosis are complex and still incompletely understood, but it is assumed that different localization of death receptors, ability of *p53* to regulate translation or the direct role of *p53* in the mitochondria are involved (37). It is understood that pathways of apoptosis, in which *p53* is a central hub, are activated. One established pathway involves increasing gene expression of pro-apoptotic stimuli, e.g. Bcl-2 family members as BCL2-Associated X Protein (*BAX)*. Additionally, one of the principal responsive genes of *p53* is *CDKN1A* (cyclin-dependent kinase inhibitor 1A), also known as *p21,* which is involved in growth arrest.

1.5 Micronuclei and DNA damage

During normal cell division, chromosomes will be divided between two daughter cells which will each contain one nucleus. In some circumstances, a micronucleus will be formed during the anaphase of mitosis or meiosis. This is a small nucleus, consisting of a chromosome or a fragment of a chromosome, which is not incorporated in the newly formed nuclei after mitosis of meiosis. This means that after cell division, one daughter cell ends up with one nucleus and the other ends up with one large and one small nucleus, called micronucleus (42).

It has been shown in epidemiological research that environmental (e.g. smoking) and genetic influences (e.g. age) can cause spontaneous micronuclei (MN) formation after causing chromosomal damage (43, 44). MN frequency is a well established biomarker for chromosomal breakage, instability or DNA damage and hence is often used in tests for genotoxicity assessment of chemical components. There is a chance that more than one micronucleus is formed when more genetic damage occurs. Furthermore, increased MN frequency is linked to increased risk of cancer and cardiovascular diseases (45, 46). MN frequencies may serve as a phenotypic anchor for genotoxicity (47).

Van Leeuwen and colleagues studied the impact of exposure to environmental air pollution on children at the transcriptional level with genome-wide oligonucleotide microarrays. This gave insight into the genome-wide response to environmental exposures (48). Investigation at transcriptional level gives a better understanding of the modulation of biological pathways and processes as a result of environmental stress. Different gene expression patterns were found between the differently exposed populations. Subsequently, these gene expression data were correlated with MN frequencies, in order to associate individual gene expression with an established biomarker of effect that is representative for increased genotoxic risk. Children exposed to higher concentrations of air pollution, showed a significantly higher

average MN frequency. Furthermore, Van Leeuwen et al. developed a MN formation network based on a priori knowledge retrieved from literature by using the pathway tool MetaCore (GeneGo, San Diego, CA, USA). The gene network contained 27 genes and three gene complexes that are related to processes involved in MN formation, e.g. spindle assembly checkpoint, cell cycle checkpoint and aneuploidy. In the MN network, *p53* was represented as the major hub, a central node through which many connections go. The MN-related gene network was tested against the transcriptomics case study of air pollution exposure associated with MN measurements. Van Leeuwen and colleagues concluded that six genes of the network were regulated or associated with MN frequencies and PM-exposure; *BAX*, *DNMT1*, *PCNA*, *HIC1*, *p21* and *CDC20* (Figure 1). Based on these six genes, and in combination with *p53* and *IL-6*, a dedicated network was created (49). This network is possibly suited for screening of populations for MN formation.

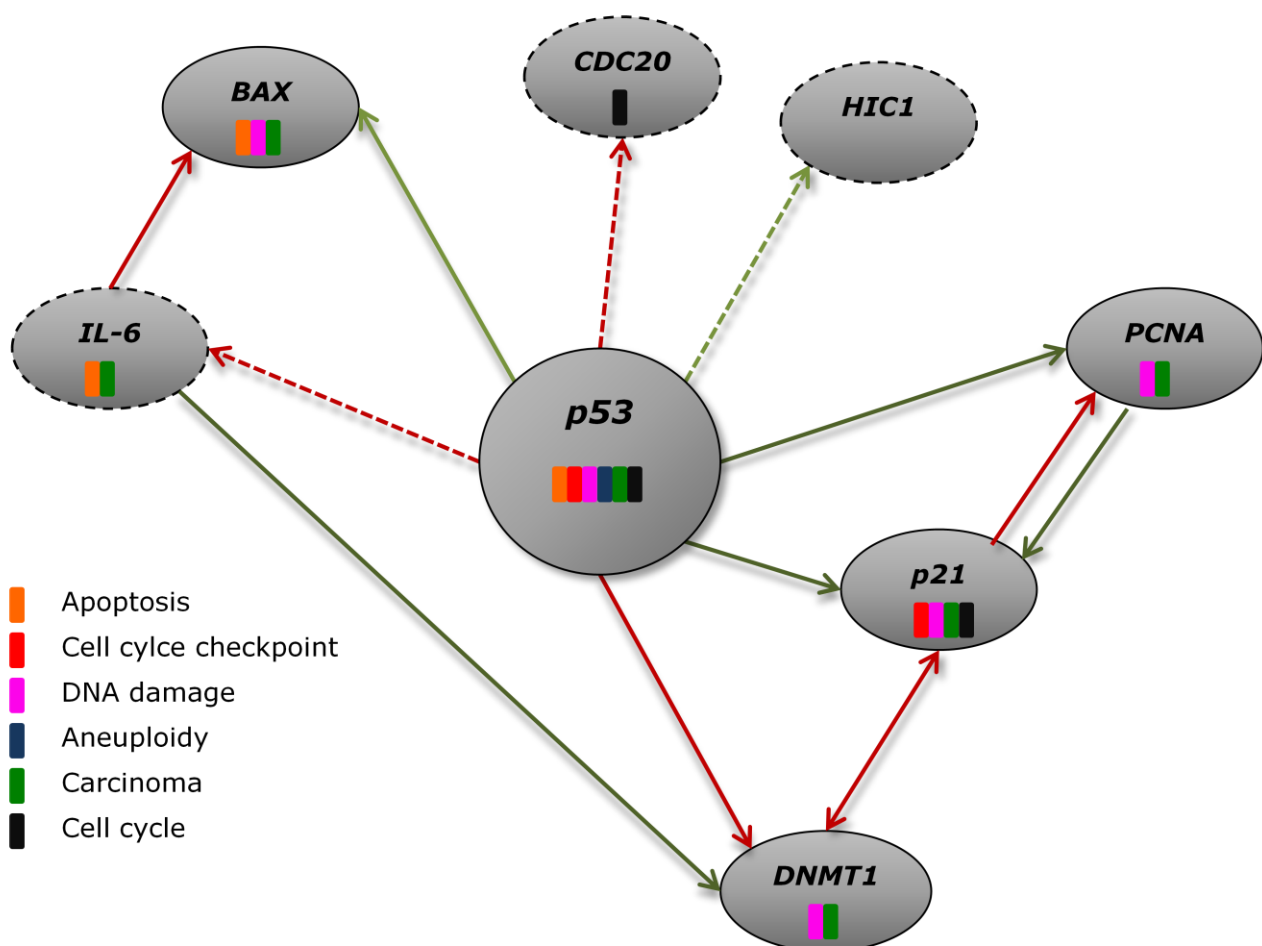

Figure 1: Molecular network MN-related genes. Activation (green) and inhibition (red) effects are shown. Information on the genetic pathways and the biological processes the genes are involved in is added as different colours. Genes that were not investigated in this study are encircled in dotted circles. Figure was adapted from Van Leeuwen et al. (2011).

1.6 ENVIR*ON*AGE project

The ENVIR*ON*AGE project is an ongoing birth cohort that has been set up in the Centre for Environmental Sciences of Hasselt University which investigates the association between *in utero* environmental exposures and development of diseases later in life. ENVIR*ON*AGE is an acronym that emphasizes the ENVIR*ON*mental influence on the AGEing process. The project uses foetal and maternal matrices to investigate associations between PM exposure and adverse health outcomes on molecular and clinical levels.

The hypothesis of this study, which lies within the ENVIR*ON*AGE project, is that gene expression is altered in association with *in utero* PM exposure. The first objective is to investigate RNA quality and to find proper housekeeping genes for normalization of gene expression data in cord blood. The second objective is to measure gene expression of *p53* in cord blood retrieved from newborns. We will investigate the association between *in utero* PM exposure and expression of *p53*, which is a central hub in many damage-response pathways and MN formation. The third objective is to measure other target genes involved in MN formation, since this is an established biomarker of effect that is representative for an increased genotoxic risk. Selection of these genes is based on the MN formation network created by Van Leeuwen and colleagues. Lastly, we performed a pilot study to determine the usage of placental tissue for gene expression analysis in future experiments.

This research will give a more complete understanding of the molecular pathways and biological processes underlying DNA damage caused by environmental air pollution during *in utero* life.

2. Materials and methods

2.1 Study population

The ENVIR*ON*AGE birth cohort was set up in 2010 in cooperation with the Hospital South-East Limburg (ZOL) in Genk, Belgium. Pregnant women, who intended to deliver in ZOL, were informed about the study and asked to participate. They all signed an informed consent. After the delivery, mothers had to fill out questionnaires concerning lifestyle factors such as age, maternal education, smoking status, ethnicity, place of residence, pre-gestational body mass index (BMI) and parity. In addition, perinatal parameters of the newborn such as sex, birth weight and length, birth date, gestational age, Apgar score and ultrasonographic data were collected. All the procedures in this study were approved by the Ethical Committee of Hasselt University and ZOL.

2.2 Collection and processing of cord blood samples

2.2.1 Cord blood collection

Immediately after the baby was born, umbilical cord was clamped and cord blood collected. Samples projected for RNA analysis were collected in RNA stabilizing tubes; either into Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA) or PAXgene Blood RNA Tubes (BD PreAnalytical Systems, Franklin Lakes, NJ).

PAXgene Blood RNA Tubes contain a reagent that immediately stabilizes intracellular RNA and reduces RNA degradation. PAXgene Blood RNA tubes (space for 2.5 mL blood) were mixed very well after collection and gradually cooled. They were first stored at room temperature for 20 till 72 hours, afterwards at -20°C for at least 24 hours and finally at -80°C for long storage. Tempus Blood RNA tubes were mixed very well by vortex and immediately stored at -80°C. Tempus Blood RNA Tubes contain 6 mL stabilizing reagent, which will mix with the blood (3 mL) after this is drawn into the tube. Lysis will occur almost immediately and the stabilizing reagent will inactivate cellular RNases and precipitate RNA and genomic DNA while proteins will remain in the solution.

2.2.2 RNA extraction

Tempus tubes were extracted with Tempus spin RNA isolation Reagent kit (Life Technologies, CA, USA). Before starting the extraction, it was important to check the volume of blood in the collection tubes and to examine the structure, since this can affect the results. The extraction was performed according to the manufacturer's guidelines. RNA was eluted in 70 µl nucleic acid purification elution solution and stored in -80°C. PAXgene tubes were extracted using the PAXgene blood miRNA kit (Qiagen, Valencia, CA, USA). Extraction was performed according to the manufacturer's protocol, eluted in 80 µl elution buffer, and stored in -80°C.

2.3 Determination of RNA quality

RNA concentration was measured using Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, the Netherlands). RNA purity was estimated by examining the ratio of absorbance at 260/280 and at 260/230.

RNA integrity and quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies). The principle of bioanalyzer is based on capillary gel electrophoresis, where RNA-fragments are separated by size. The chip of the Agilent-kit consists of a network of micro channels and reservoirs. The channels are filled with gel matrix and the reservoirs with the samples which makes it possible to do electrophoresis on miniature scale. Only 1 µl sample is necessary for 1 analysis. Analysis was performed according to the protocol provided by the manufacturer and gives a RIN (RNA integrity number)-value. RIN-value is a number from 1 to 10, with 1 being the most degraded and 10 being the most intact, which gives an estimation of total RNA sample integrity. The number is not based on the ratio of ribosomal bands, but on the entire electrophoresis trace of the RNA sample (50).

2.4 Gene expression

Primer efficiency of eleven selected candidate housekeeping genes and five target genes was assessed on test samples. Selection of these genes, shown in Table 2, was based on literature. Afterwards, a Genorm analysis was performed to determine the most stable reference genes in cord blood since normalization of quantitative real-time polymerase chain reaction (qPCR) is important to obtain accurate gene expression data. Subsequently, gene expression of five MN-related target genes was measured.

2.4.1 cDNA synthesis

First, RNA derived from umbilical cord blood was converted to cDNA (complementary DNA) using the GoScript™ Reverse Transcription System kit (Promega, Wisconsin, United States). An RNA input of maximum 5 µg was used for cDNA synthesis. cDNA synthesis was performed according to the manufacturer's guidelines and stored at -20°C until use.

2.4.2 Primer efficiency

Primer efficiency and optimal concentration of cDNA input for qPCR were determined using a dilution series of test samples of cord blood. These two test samples consisted of a mix of ten samples collected from Tempus tubes and ten samples collected from PAXgene tubes. Starting with an input of 20 ng/µl cDNA, a dilution series was made as followed: 1,1/3,1/9,1/27,1/81,1/243. QPCR was performed on the dilution series for every gene expression assay as described in section 2.4.3 of materials and methods. The average C_T-value for every dilution was calculated and a plot of C_T vs. log cDNA dilution concentration was constructed. Amplification efficiency was determined based on the slope of the standard curve using the following equation: $E\%=[10^{(-1/slope)}-1]\%$. Amplification efficiency was calculated for PAXgene and Tempus samples separately for each gene.

Table 2: Candidate reference and target genes with assay ID from IDT (Integrated DNA Technologies, USA).

	Full name	Assay ID
Reference genes		
HPRT1	hypoxanthine phosphoribosyltransferase 1	Hs.PT.53a.20881146
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	Hs.PT.39a.22214858
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	Hs.PT.56a.25515089
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs.PT.53a.24391631.gs
B2M	beta-2-microglobulin	Hs.PT.39a.22214854
UBC	ubiquitin C	Hs.PT.39a.22214853
ACTB	actin, beta	Hs.PT.39a.22214847
RPLP0	ribosomal protein, large, P0	Hs.PT.56a.40434846
TBP	TATA box binding protein	Hs.PT.56a.20792004
CYC1	cytochrome c-1	Hs.PT.56a.20696349.gs
IPO8	importin 8	Hs.PT.56a.40532361
Target genes		
CDKN1A (p21)	cyclin-dependent kinase inhibitor 1A	Hs.PT.58.40874346.g
PCNA	proliferating cell nuclear antigen	Hs.PT.58.1446155
DNMT1	DNA (cytosine-5-)-methyltransferase 1	Hs.PT.58.26259414.gs
BAX	BCL2-associated X protein	Hs.PT.56a.19141193.g
p53	tumour protein *p53*	Hs.PT.58.39489752.g

2.4.3 Quantitative real-time polymerase chain reaction

qPCR was set up in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA, USA) in which 2 µl of every sample was supplemented with 8 µl master mix consisting of 0.5 µl 20xTaqman gene expression assay, 5 µl 2xTaqman Fast advanced master mix and 2.5 µl RNase free water. Assay ID of every Taqman gene expression assay can be found in Table 2. All samples were run in triplicate with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and a non-template control was used in every assay. The following thermal cycling conditions were used: 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C for and 1 minute at 60°C. After the run, expression levels were determined as quantification cycles, which were expressed as C_T (cycle threshold)-values. C_T-values were analyzed using SDS2.3 software and deviating triplicates were removed.

2.4.4 Genorm analysis

For stability comparisons of candidate reference gens, a Genorm analysis was performed. First, a qPCR assay was conducted for every candidate reference gene with cDNA samples diluted to 3 ng/µl since this input concentration showed the best efficiency (see section 2.4.3 of materials and methods). Subsequently, Qbase (Biogazelle, Zwijnaarde, Belgium), a software used to examine data of all types of qPCR experiments, was used to determine the most stable and best combination of reference genes (51). Qbase has an integrated Genorm analysis which compares every gene pair-wise to all the other tested genes and calculates the average expression stability and the pair-wise variation for a given gene, respectively expressed as M-value and V-value. Stepwise exclusion of the gene with the highest M-value allows ranking of the tested genes according to their expression stability. V-value indicates the

acceptable stability of the reference gene combination and will determine the benefit of adding extra reference genes for the normalization process (52).

2.4.5 Gene expression of target genes

The mRNA expression levels of five target genes (Table 2) involved in a MN formation pathway were analyzed in 96 cord blood samples by qPCR assay (see section 2.4.3 of materials and methods). Qbase software (Biogazelle, Zwijnaarde, Belgium) was used to determine relative gene expression, which was normalized by the combined expression of three stable reference genes specifically chosen for cord blood: *TBP*, *CYC1* and *IPO8*. Qbase uses a modification of the classic delta-delta-C_T method and takes multiple reference genes into account and uses inter-run calibration algorithms to correct for run-to-run differences (51).

2.5 Exposure Assessment

Regional background levels of $PM_{2.5}$ for each mother's home address were calculated using a kriging spatial temporal interpolation method that uses land cover data obtained from satellite images (Corine land cover data set) in combination with monitoring stations (n=34). This model provides interpolated $PM_{2.5}$ values from the Belgian telemetric air quality networks in 4 × 4 km grids (53). Different windows of exposure during pregnancy for each mother individually were calculated. Trimesters were defined as: 1–13 weeks (trimester 1), 14–26 weeks (trimester 2) and 27 weeks to delivery (trimester 3).

2.6 Statistical analysis

Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). The Shapiro-Wilk test was used to test for normality and gene expression data were log10 transformed, which resulted in a satisfactory approximation to a normal distribution. Spearman correlation coefficients and multiple linear regression were applied to determine associations between $PM_{2.5}$ exposure and gene expression. Reported *p*-values are two-sides and *p*-values <0.05 were considered significant. In addition, we performed multivariate mixed effect models which we used to investigate the association between $PM_{2.5}$ exposure and multiple target genes of the MN formation network. Before entering these target genes into the mixed effect model, we first calculated z-scores for each gene individually to put them on the same scale. The z-score, which is also called the standard score, uses the population mean and standard deviation to standardize or normalize sample values so that they share a common underlying distribution.

3. Results

3.1 Population characteristics and exposure levels

Demographic and prenatal characteristics of the 96 mother-newborn pairs included in this study are summarized in Table 3. Maternal age averaged 29.4 years and ranged from 21 to 42 years. Mean (± SD) pre-gestational BMI of the participating mothers was 24.7 (± 4.6) kg/m^2. Smokers were defined as having smoked before the pregnancy (17.7%), and smoked before and during the pregnancy (20.8%). We used maternal education as a marker of socioeconomic status and encoded this as low (no diploma or primary school, 8.3%), middle (high school, 37.5%) or high (college or university degree, 54.2%). The study population included 46 male newborns, and 88.5% (*n*=85) of the newborns were classified as European. Newborns had an average gestational age of 39.3 (± 1.1) weeks and none of the newborns were preterm. Of the newborns, 24.0% (*n*=23) was delivered in a cold period (September until March) and 76.0% (*n*=73) was delivered in a warm period (March until September).

$PM_{2.5}$ exposure characteristics are displayed in Table 4 and averaged 17.7 (± 6.9) µg/m^3 during the first trimester of pregnancy, 19.3 (± 5.3) µg/m^3 during the second trimester and 16.9 (± 6.6) µg/m^3 in the third trimester.

Table 3: Study population characteristics (*n*=96)

Maternal		Newborn	
Characteristic	**Mean± SD or n (%)**	**Characteristic**	**Mean± SD or n (%)**
Age (years)	29.4 ± 4.2	Gestational age (weeks)	39.3 ± 1.1
< 25	14 (14.6)	Sex	
25-35	72 (75.0)	Male	46 (47.9)
≥ 35	10 (10.4)	Female	50 (52.1)
Pre-gestational BMI (kg/m^2)	24.7 ± 4.6	Ethnicity	
Maternal education		European	85 (88.5)
Low	8 (8.3)	Non-European	11 (11.5)
Middle	36 (37.5)	Season	
High	52 (54.2)	Cold period	23 (24.0)
Smoking		Warm period	73 (76.0)
Never	59 (61.5)		
Before pregnancy	17 (17.7)		
Before and during pregnancy	20 (20.8)		
Parity			
1	43 (44.8)		
2	43 (44.8)		
≥ 3	10 (10.4)		

Table 4: Exposure characteristics

Pollution indicator	Mean ±SD	25th percentile	75th percentile
$PM_{2.5}$ (µg/m^3)			
Trimester 1	17.7 ± 6.9	12.1	21.7
Trimester 2	19.3 ± 5.3	15.3	23.5
Trimester 3	16.9 ± 6.6	11.3	22.1

3.2 RNA quality in cord blood

RNA yield, purity and integrity were compared between test samples of umbilical cord blood collected in Tempus Blood RNA tubes (n=9) and PAXgene blood RNA tubes (n=9). Results are displayed in Figure 2; the RIN value was consistently higher in samples collected in PAXgene tubes, but yield and purity was on average higher in samples collected in Tempus tubes.

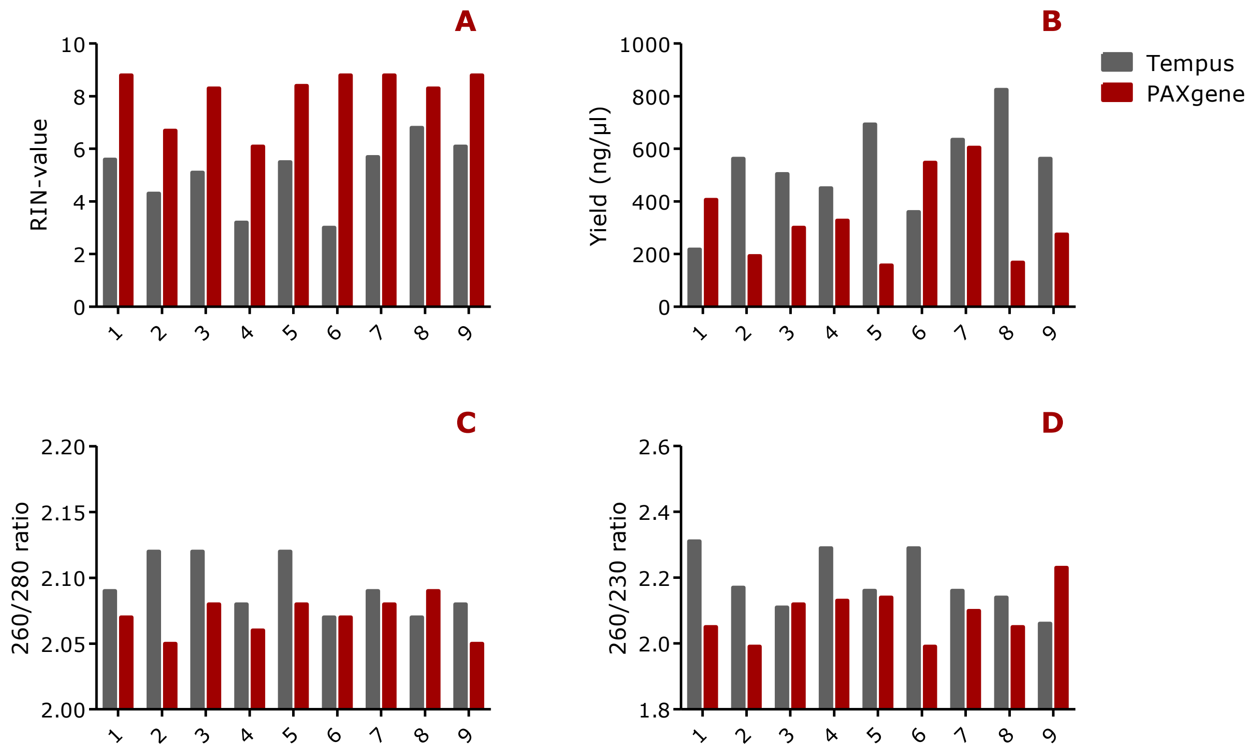

Figure 2: Comparison of RNA quality between RNA extracted from Tempus and PAXgene blood RNA tubes. RIN-value (A), yield (ng/µl) (B), and purity (C and D) of 9 test samples is displayed on x-axis. Purity was measured as ratio of absorbance at 260/280 (C) and 260/230 (D).

In addition, RNA was extracted from 151 cord blood samples collected in Tempus blood RNA tubes. RNA integrity was measured and a RIN-value of 6 was used as a cut-off value for suitable RNA samples, which led to a final sample size of 96 for gene expression analysis. Mean RNA yield was 516 ng/µl ± 282 and average RIN-value of 96 selected samples was 8.17 ± 0.88.

3.2.1 Pilot study for RNA quality in placental tissue

This project fits within the bigger ENVIR*ON*AGE birth cohort, which also uses placental tissue as a matrix. A pilot study was set up to determine RNA quality in placental tissue, since gene expression analysis will also be performed on placental tissue in future experiments.

Placental tissue was collected on four different time points after delivery (0h, 24h, 48h, 72h) from which RNA was extracted. Results showed that placental tissue should be collected within 24 hours after delivery to obtain high-quality RNA for gene expression analysis (Figure 3). Detailed information about the methods and results can be found in supplement A.

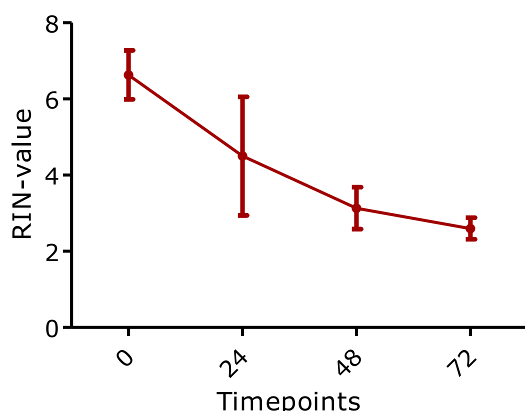

Figure 3: RIN-values of RNA in placental tissue on four different time points after delivery; T0, T24, T48 and T72. RNA quality decreases rapidly with time. RIN-value should at least be 6 to have RNA of sufficient quality for further gene-expression analysis. Results are displayed as mean with standard deviation of the sampling distribution (*n*=4).

3.3 Gene expression in cord blood

3.3.1 Primer efficiency of reference and target genes

Primer efficiency of eleven candidate reference genes and five target genes was determined with qPCR and is displayed in Table 5. Efficiency is acceptable between 90% and 110%, which is the case for all genes. Efficiency curves showed that an input of 3 ng/µl is optimal for every gene used in this study. In general, no difference in efficiency was observed when using cDNA derived from cord blood that was collected in Tempus blood RNA or PAXgene blood RNA tubes. In future research, we will also measure gene expression in placental tissue, so efficiencies on fresh placental tissue were also determined with the same method. Detailed results can be found in supplement B.

Table 5: Efficiency (%) of primer assay of eleven candidate reference genes and five target genes measured on cord blood samples collected in Tempus and PAXgene blood RNA tubes.

		Tempus	**PAXgene**
Reference genes			
	ACTB	94.4	94.5
	B2M	101.2	104.3
	CYC1	97.3	102.0
	HPRT1	100.1	99.7
	IPO8	108.5	110.6
	POLR2A	99.4	102.6
	RPLP0	99.8	99.4
	TBP	102.6	104.5
	UBC	101.9	104.6
	YWHAZ	102.4	99.3
	GAPDH	93.7	94.1
Target genes			
	BAX	94.7	91.5
	P21	94.9	98.0
	DNMT1	100.5	92.5
	PCNA	96.8	96.7
	P53	96.6	90.1

3.3.2 Genorm analysis of reference genes

Genorm analysis was performed in Qbase on 25 samples for 11 reference genes. This analysis compares every gene pair-wise to all the other tested genes and calculates the average pair-wise variation for a given gene and generates an M-value (expression stability) and V-value (pair-wise variation). M-value, which is the average expression stability of every reference gene should be lower than 0.5 to ensure gene stability across samples. *B2M, UBC* and *RPLP0* have an M-value above 0.5, which indicates these will not be stable reference genes. *POLR2A, ACTB, TBP, IPO8, YWHAZ, CYC1, GAPDH* and *HPRT1* have an M-value below 0.5, so all these genes are valid candidate reference genes (Figure 4A). The v-value indicates the acceptable stability of a reference gene combination. An arbitrary cut off value of 0.15 indicates acceptable stability of the reference gene combination. Results show that using 2 or 3 reference genes will be sufficient (Figure 4B). More reference genes can be used, but will not provide any additional value to the normalization. Genorm analyses were also performed for samples collected in PAXgene blood RNA tubes and Tempus blood RNA tubes separately. These analyses showed no difference in stability of reference genes when using PAXgene or Tempus tubes. In separate analyses only *B2M* had an M-value above 0.5 and V-value showed for both analyses that use of 2 or 3 reference genes is sufficient.

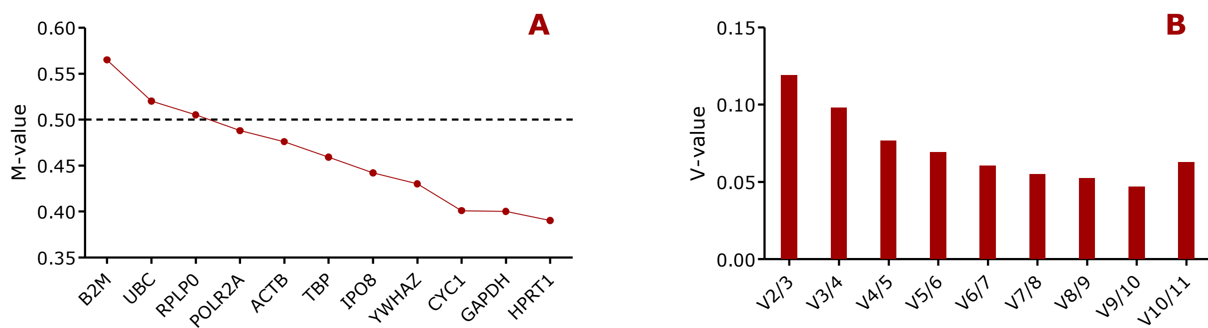

Figure 4: Selection of the most suitable reference genes for normalization in umbilical cord blood using Genorm analysis. The expression stability of candidate genes in umbilical cord blood was determined. A: Stepwise exclusion of the least stable genes by calculating the average expression stability measure M. M-value was calculated for each gene and the least stable gene with the highest M-value was automatically excluded from the next calculation round. The x-axis indicates the ranking of the genes according to their expression stability. B: Determination of the optimal number of reference gens for normalization. X-axis shows the pair-wise variation V_n/V_{n+1} between normalization factors of n and n+1 genes.

Reference genes that were finally chosen for normalization in gene expression analyses are *IPO8*, *TBP* and *CYC1*. These three genes were not the genes with the lowest M-value, but were chosen because of practical reasons. They had been commonly used in the laboratory before, and are not active in the same pathways and not co-regulated. Genorm analysis was also performed on placental samples, to determine the best combination of reference genes in fresh placental tissue. Results can be found in supplement C and these results were also taken into account when selecting the final reference genes for cord blood.

3.4 Predictors of *p53* gene expression

Gene expression of *p53* was significantly associated with gestational age (β=-0.02 ± 0.008, p=0.05) and maternal education (β=-0.02 ± 0.01, p=0.05). Gender of the newborn, maternal age, mother's smoking status, season of delivery (warm or cold season), ethnicity of grandparents and parity were not significantly associated with gene expression of *p53*, but were forced into the model as co-variables.

3.5 *P53* gene expression in association with *in utero* $PM_{2.5}$ exposure

Unadjusted analysis showed that $PM_{2.5}$ exposure during the first trimester is significantly associated with gene expression of *p53* in cord blood (Figure 5, r=0.24, p=0.02). $PM_{2.5}$ exposures during the second and third trimester of the pregnancy were not significantly associated with gene expression of *p53* (respectively r=-0.15, p=0.15 and r=-0.14, p=0.17). Analyzing the data with or without the outlier in trimester 1 (X=50.5, Y=0.02) did not change the results.

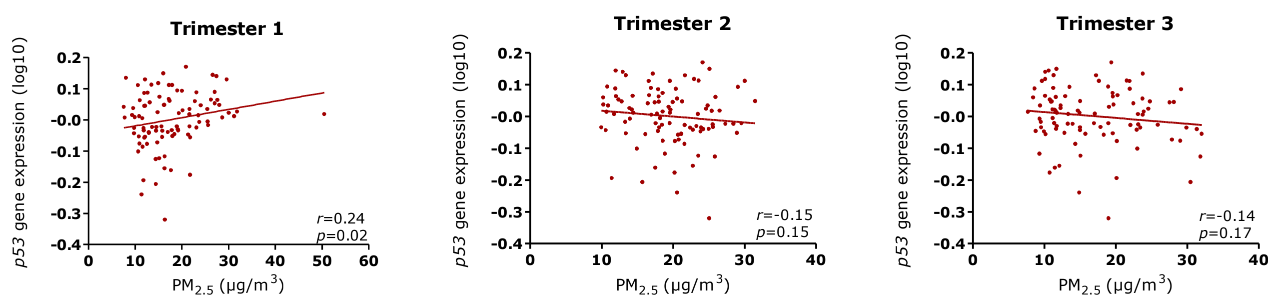

Figure 5: Association between *p53* gene expression in cord blood and $PM_{2.5}$ exposure during pregnancy. Y-axis shows the gene expression values of *p53*, which were log10 transformed. X-axis shows $PM_{2.5}$ exposure of the mother in µg/m^3. Spearman correlation coefficient and p-value are displayed for every association.

In multivariate analysis, the linear regression model was adjusted for newborn's gender, maternal age, gestational age, mother's smoking status, season of delivery, maternal education, ethnicity and parity. The expression of *p53* remained positively associated with $PM_{2.5}$ exposure during the first trimester (Figure 6). We observed an increase of 3.21% (95% CI: 0.41 to 6.01, p=0.03) in gene expression of *p53* for each increase of 5 µg/m^3 of $PM_{2.5}$ exposure. Exposure during the second and third trimester of the pregnancy were not significantly associated with altered gene expression levels of *p53* in cord blood (respectively -0.46%, 95% CI: -5.24 to 4.33, p=0.85 and -2.49%, 95% CI: -5.67 to 0.69, p=0.13)

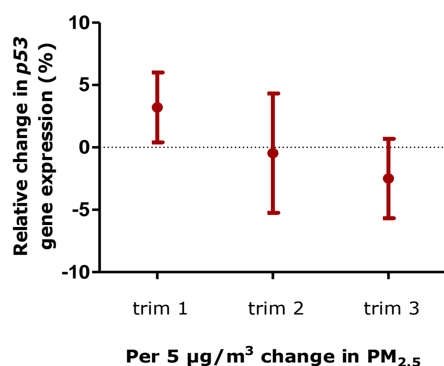

Figure 6: The relative change in gene expression of *p53* (95% CI) in cord blood for each 5 µg/m^3 increase of $PM_{2.5}$ exposure. Changes in gene expression of *p53* are shown for the first, second and third trimester of the pregnancy. The model was adjusted for newborn's gender, maternal age, gestational age, mother's smoking status, season of delivery, maternal education, ethnicity and parity. Values of *p53* gene expression are log10 transformed.

3.6 *P53* gene expression is linked to other target genes involved in micronuclei formation

Table 6 displays the associations between *p53* and the different target genes in the molecular network as described by Van Leeuwen et al.(49). *P53* shows a significant association with gene expression of *DNMT1* (r=0.26, p=0.01) and modest associations with *BAX* (r=0.19, p=0.06) and *PCNA* (r=0.18, p=0.08). After adjusting for newborn's gender, maternal age, gestational age, mother's smoking status, maternal education, ethnicity and parity in a linear regression model, gene expression of *p53* was still modestly associated with gene expression of *BAX* (β=0.12, p=0.05) and *PCNA* (β=0.12, p=0.07) and significantly associated with *DNMT1* (β=0.38, p<0.001). Gene expression of *p53* was not significantly associated with expression of *p21* in the unadjusted (r=-0.07, p=0.50) or adjusted model (β=-0.04, p=0.60). The residuals of these models showed a normal distribution.

Significant associations were also found within the molecular MN formation network (Table 6). Gene expression of *BAX* was significantly associated with gene expression of *PCNA* (r=0.28, p=0.007) and *DNMT1* (r=0.25, p=0.01). Subsequently, a significant association was found between gene expression of *p21* and gene expression of *DNMT1* (r=-0.28, p=0.005) and *PCNA* (r=-0.35, p=0.0005).

Table 6: Associations between genes involved in the MN formation network. Spearman correlation coefficient, *p*-value and number of subjects are displayed.

		p53	***BAX***	***DNMT1***	***PCNA***	***p21***
p53	*r*	1	0.19	0.26	0.18	-0.07
	p		0.06	0.01	0.08	0.50
	n	96	95	96	96	95
BAX	*r*	0.19	1	0.25	0.28	-0.05
	p	0.06		0.01	0.007	0.63
	n	95	95	95	95	94
DNMT1	*r*	0.26	0.25	1	0.35	-0.28
	p	0.01	0.01		0.0005	0.005
	n	96	95	96	96	95
PCNA	*r*	0.18	0.28	0.35	1	-0.35
	p	0.08	0.007	0.0005		0.0005
	n	96	95	96	96	95
p21	*r*	-0.07	-0.05	-0.28	-0.35	1
	p	0.50	0.63	0.005	0.0005	
	n	95	94	95	95	95

3.6.1 Multivariate mixed effect model

Given the knowledge that *p53* acts as a central hub for activating other genes involved in MN formation, a multivariate mixed effect model in which all target genes of *p53* were combined into one outcome with *p53* as the independent variable was performed. Different genes were interpreted as repeated measurements and z-scores were applied to put the expression levels on the same scale. Results showed a significant association between an increase of *p53* gene expression and an increase in outcome of four target genes in an unadjusted (β=2.04, p=0.001) and adjusted model (β=2.20, p=0.002). The model was corrected for the same covariates as aforementioned. The interaction term was not significant for the unadjusted (p=0.18) or adjusted (p=0.17) model, suggesting that *p53* influences all target genes equally.

3.7 *In utero* $PM_{2.5}$ exposure and the molecular network of micronuclei-related genes

Spearman correlation coefficients between *in utero* $PM_{2.5}$ exposure in different trimesters and the individual genes involved in MN formation network are presented in Table 7. Only *p53* gene expression was significantly associated with exposure to $PM_{2.5}$ in trimester 1 as mentioned earlier (r=0.24, p=0.02). $PM_{2.5}$ exposure during the first, second or third trimester had no significant effect on the gene expression of *BAX*, *PCNA*, *DNMT1* or *p21* directly.

Table 7: Association of $PM_{2.5}$ exposure during pregnancy with expression of different target genes in cord blood. Spearman correlation coefficient and *p*-value are displayed.

$PM_{2.5}$		*p53*	*BAX*	*PCNA*	*DNMT1*	*p21*
Trimester 1	*r*	0.24	-0.07	-0.16	-0.03	0.06
	p	0.02	0.49	0.13	0.78	0.56
Trimester 2	*r*	0.15	0.09	-0.11	0.05	-0.003
	p	0.15	0.38	0.28	0.63	0.98
Trimester 3	*r*	-0.14	0.08	0.006	0.02	0.03
	p	0.17	0.46	0.95	0.87	0.79

Subsequently, a multivariate mixed effect model in which we combined the MN genes as one outcome (*p53*, *BAX*, *DNMT1*, *PCNA* and *p21*), was used to determine the association between exposure to $PM_{2.5}$ and the MN formation network (Table 8). No significant association was found with $PM_{2.5}$ exposure in each trimester in unadjusted analysis ($p \geq 0.34$). However, in adjusted analysis, we observed a modest positive association in trimester 1 (β=0.01, p=0.09). The interaction term was significant in trimester 1 for adjusted (p=0.03) and unadjusted analysis (p=0.005) indicating a different response of $PM_{2.5}$ exposure on the separate genes in the MN formation network. Next, we performed an analysis with only genes that individually correlated positively with the MN formation (*p53, BAX*, *PCNA*, *p21*), as described in literature. Unadjusted and adjusted analysis still did not show a significant association with $PM_{2.5}$ exposure, although a modest positive association in trimester 1 was observed (Table 8). The interaction term in trimester 1 of unadjusted (p=0.003) and adjusted (p=0.02) analysis remained significant.

Table 8: Associations of $PM_{2.5}$ exposure during pregnancy with the MN formation network. Estimate, *p*-value and interaction term are displayed.

		MN formation network (*p53, BAX, DNMT1, PCNA, p21*)		**MN formation network (*p53, BAX, PCNA, p21*)**	
$PM_{2.5}$		Model[a]	Model[b]	Model[a]	Model[b]
Trimester 1	β	0.006	0.01	0.005	0.01
	p	0.34	0.09	0.40	0.11
	p interaction	0.005	0.03	0.003	0.02
Trimester 2	β	-0.004	0.0009	-0.008	-0.004
	p	0.65	0.94	0.44	0.76
	p interaction	0.21	0.20	0.35	0.44
Trimester 3	β	-0.003	-0.006	-0.001	-0.006
	p	0.70	0.47	0.87	0.49
	p interaction	0.48	0.69	0.40	0.57

[a] Model is unadjusted [b] Model is adjusted for newborn's gender, maternal age, gestational age, mother's smoking status, maternal education, ethnicity and parity.

4. Discussion

The aim of this study was to investigate expression of genes involved in a MN formation network in association with PM exposure during pregnancy. The key finding of our study is that *p53* gene expression in cord blood was significantly associated with *in utero* $PM_{2.5}$ exposure during the first trimester of pregnancy. Furthermore, modest associations were found between gene expression of *p53* and expression of target genes involved in the molecular MN network. No direct significant associations were found between $PM_{2.5}$ exposure and the genes in the network individually, neither between $PM_{2.5}$ exposure and the entire MN formation network, although a positive trend was observed during trimester 1. Taken together, we postulate that exposure to PM affects *p53* gene expression in cord blood. In this way *p53* acts as a central hub for the regulation of MN formation or other responsive mechanisms associated with cellular damage that we have not investigated.

4.1 *P53* as a central hub for micronuclei formation

Van Leeuwen et al. published a network of genes involved in MN formation with *p53* as a central hub (49). It has been established in literature that *p53* is a tumour suppressor gene, which plays an important role in cellular response to a variety of environmental and intracellular stresses (37). Different stress signals, have been demonstrated to increase *p53* levels (54). *P53* activation involves an increase in *p53* protein levels as well as structural changes in the protein. Post-translational modifications generally result in *p53* stabilization and accumulation in the nucleus, where *p53* interacts with sequence-specific sites on its target genes (55, 56). This process will activate signalling networks such as the MN formation network proposed by Van Leeuwen et al., in which *p53* is the central hub. The direct relationship between *p53* induction and MN formation has been reported in experimental research (57). The genes that we investigated in this study and that are involved in the MN network are *BAX*, *DNMT1*, *PCNA* and *p21*.

The protein encoded by *BAX* is a member of the BCL2 protein family which forms a dimer with BCL2, and acts as a pro-apoptotic regulator. It can cause release of cytochrome c by interaction with the mitochondrial voltage-dependent anion channel, which will trigger apoptosis. Expression of this gene is regulated by tumour suppressor *p53* and has been shown to be involved in *p53*-mediated apoptosis. Up-regulation of *BAX* is significantly correlated with increased MN frequency (49). *DNMT1* is important in the regulation and establishment of tissue-specific patterns of methylated cytosine residues. *DNMT1* will associate with DNA replication sites in the S-phase to maintain the methylation pattern in newly synthesized strands and it will associate with chromatin during G2 and M phases to maintain DNA methylation independently of replication. Published research within our research group showed that global DNA methylation patterns in placental tissue are altered in association with *in utero* exposure to particulate air pollution (30). Alteration of methylation pattern in cord blood has not yet been confirmed, but changes in gene expression of *DNMT1* can be indicative of these alterations. *DNMT1* levels have been shown to be negatively correlated with MN frequency (49). The encoded protein of *PCNA* helps to increase the processivity of the leading strand synthesis during RNA replication. The protein is ubiquitinated in response to DNA damage and is involved in the RAD6-dependent DNA repair pathway. Findings of Xu and colleagues suggest a complex cellular response to DNA damage in which *p53* transiently activates expression of *PCNA* in order to obtain limited DNA repair (58). *PCNA* was up-regulated in association with lower MN frequencies (49). *P21* encodes a universal inhibitor of cyclin-

dependent kinases and therefore is a regulator of cell cycle progression at the G1 phase. The expression of the gene is known to be tightly associated with *p53* expression, but this was not confirmed in our study (49). Up-regulation of *p21* was significantly correlated with increased MN frequencies (49).

A first noteworthy result in our study is the positive association between *p53* expression and expression of the target genes *BAX*, *DNMT1* and *PCNA* in cord blood. These associations were more pronounced after adjusting for newborn's gender, maternal age, gestational age, mother's smoking status, maternal education, ethnicity and parity. As in the study of Van Leeuwen et al., we found an up-regulation of *BAX* and *PCNA* in association with an increase in *p53* gene expression. Our positive association between *p53* and *DNMT1* was not consistent with the inverse association described by Van Leeuwen et al (49). Combining the four target genes as one outcome in a multivariate mixed effect model showed a significant positive association with *p53* gene expression in cord blood. The interaction term was not significant in adjusted and unadjusted models, suggesting a common response of *p53* on all four target genes.

Furthermore, significant associations were found within the molecular network of MN formation. Consistent with the findings of Van Leeuwen et al., we found a significant inverse association between *p21* expression and expression of *PCNA* and *DNMT1* and a significant positive association between gene expression of *PCNA* and *DNMT1*. Inconsistent with the work of Van Leeuwen et al. was the positive association between expression of *BAX* and expression of *PCNA* and *DNMT1*, since Val Leeuwen dit not report any significant association. These observations found within the network are not all biologically logical and not confirmed in literature, thus should be elucidated in further experiments.

Taken together, results from our study confirm that *p53* is a central hub, which activates or represses target gene transcription of MN-related genes. These modulation effects may occur through ligand-activated mechanisms, receptor-mediated mechanisms or by interference in cell cycle control (59).

4.2 Micronuclei formation network in association with environmental stressors in cord blood

4.2.1 Phenotypic micronuclei frequency and environmental stressors

MN frequency has been reported as a phenotypic endpoint that reflects chromosomal breakage caused by exposure to genotoxic carcinogens. Correlations between genotoxic exposures, including PAHs, and MN frequency have been reported (60). Furthermore, an association between PM exposure and MN frequency in peripheral blood T-lymphocytes has been shown (61). An extensive study in Serbia showed a significant increase of MN in cord blood of newborns, born 12 months after an major environmental pollution in Kragujevac (city in central Serbia). In a follow-up study, MN frequency was measured in lymphocytes of newborns born seven years after the pollution. MN frequencies were estimated by application of cytokinesis-block micronucleus test. The cytokinesis-block micronucleus assay is the preferred method for measuring MN in cultured cells. The results showed that the mean value of MN was significantly decreased in newborns born seven years after the pollution in comparison with the newborns born 12 months after contamination (62). Correlation between MN frequency and smoking has only been reported once, but most reports do not find a significant association. In addition, associations between chromosomal damage and presence of MN are well documented. MN frequency is associated with level of chromosomal breakage (63).

4.2.2 p53 gene expression in association with in utero *$PM_{2.5}$ exposure*

Early life PM exposure has been associated with adverse health outcomes of the foetus, but molecular changes are still poorly understood (23). The key finding of this study is the significant association between *p53* gene expression in cord blood and *in utero* $PM_{2.5}$ exposure during the first trimester of pregnancy. We observed no significant association during the second and third trimester of the pregnancy. This association persisted after adjustment of newborn's gender, maternal age, gestational age, mother's smoking status, season of delivery, maternal education, ethnicity and parity.

It is known that *p53* is a transcriptional regulator, and can mediate downstream effects by the activation or repression of target genes, although mechanisms underlying these functions are still poorly understood. *P53* expression has been associated with exposure to environmental factors and chemical toxicants in experimental studies. For example, an association between benzene exposure, a chemical carcinogen, and an increase in *p53* expression has been observed in the bone marrow of mice (64). The association between PM exposure and *p53* expression has been established as well. Experimental research showed an increased *p53* protein expression and apoptosis in cells at the bronchoalveolar duct junctions in mouse lungs after PM exposure (65). Furthermore, statistically significant induction of *p53* transcripts was observed in PM-exposed human epithelial lung (L132) cells (40).

PM-induced *p53* expression can be linked to activation of DNA repair mechanisms and activation of the pathway of apoptosis. Many pathways of apoptosis may exist, but until now, only two have been elucidated in literature. An extrinsic pathway, which is represented by TNF (tumour necrosis factor)-α-induced apoptosis. Dagher and colleagues showed that PM exposure activates the TNF-α-induced pathway of apoptosis in L132 cells (40). The second pathway is the intrinsic mitochondrial pathway, in which apoptosis is triggered by the release of cytochrome c, a necessary component of non-receptor mediated-caspase activation. In the same study, it was reported that PM activates the mitochondrial pathway of apoptosis in L132 cells as well (40). Furthermore, formation of 8-hydroxy-2'- deoxyguanosine was found in the PM-exposed proliferating L132 cells, which indicates the occurrence of oxidative stress conditions. It is known that the production of ROS and the secretion of inflammatory cytokines play a role in *p53* mediated apoptosis and cell death.

Further underlying mechanisms of *p53* activation are still unclear, but formation of mutations in *p53* gene that result in alterations in gene expression are suggested (66). DNA repair efficiency in the foetus is lower than in adults, therefore the foetus is more sensitive to the effects at the level of the genome. It has been suggested that foetal exposure might lead to predisposition to develop diseases such as cancer and immune diseases during childhood or in later life possibly through modulation of the foetal transcriptome. Therefore, exposure to environmental stressors *in utero* poses a sensitive window of susceptibility.

4.2.3 Windows of susceptibility of PM exposure

In this study, an association was found between gene alteration and $PM_{2.5}$ exposure. $PM_{2.5}$ has a relatively small size and large surface area, which gives it the opportunity to access biological sites large particles are not able to reach. This may lead to functional changes of critical target sites (67). Our findings show that exposure to PM during the first trimester of pregnancy is most associated with changes in *p53*

expression in cord blood. This suggests that the first trimester might be a potential window for susceptibility to $PM_{2.5}$ exposure. It is reported that air pollution exposures in the first and third trimester of the pregnancy have the most relevance for low birth weight and preterm birth (68). The first trimester is a very critical window of exposure for the foetus, since this is the most important stage in development. Although adverse pregnancy outcomes have been consistently reported, we did not find a significant association between birth weight and PM exposure in cord blood.

4.2.4 Micronuclei formation network in association with in utero *$PM_{2.5}$ exposure*

No significant association was found between $PM_{2.5}$ exposure and gene expression of *BAX*, *PCNA*, *DNMT1* or *p21* individually in cord blood. When applying a multivariate mixed effect model to investigate the association between the entire MN formation network and $PM_{2.5}$ exposure, no significant association was found in trimester 1, 2 or 3 of the pregnancy, but a positive trend in trimester 1 was observed. However, the interaction term was significant in trimester 1, which indicates a different response of $PM_{2.5}$ exposure on different genes involved in the MN formation network. This suggests that $PM_{2.5}$ exposure will influence the MN formation network indirectly by affecting *p53* transcription. A multivariate mixed effect model was also applied with as outcome only the genes that correlated positively with MN formation as described in literature. This showed no significant effect, but also a positive trend in trimester 1 was observed. In addition, the interaction term remained significant.

The study of Van Leeuwen et al. was performed with blood derived from children living in a district with elevated levels of air pollution and children of a rural area were used as control group. In our study, cord blood derived from newborns was used to investigate gene expression. Exposure effects of PM might be more clear in blood, compared to cord blood. The effects of the exposure have to "trespass" the placental barrier to reach the foetus. This might indicate why only an alteration in *p53* transcripts in association with PM exposure is found within this study.

We propose that alteration in p53 gene expression is a "first line" response to PM exposure, and could results in changes in other MN formation genes that are not directly affected by PM exposure (Figure 7).

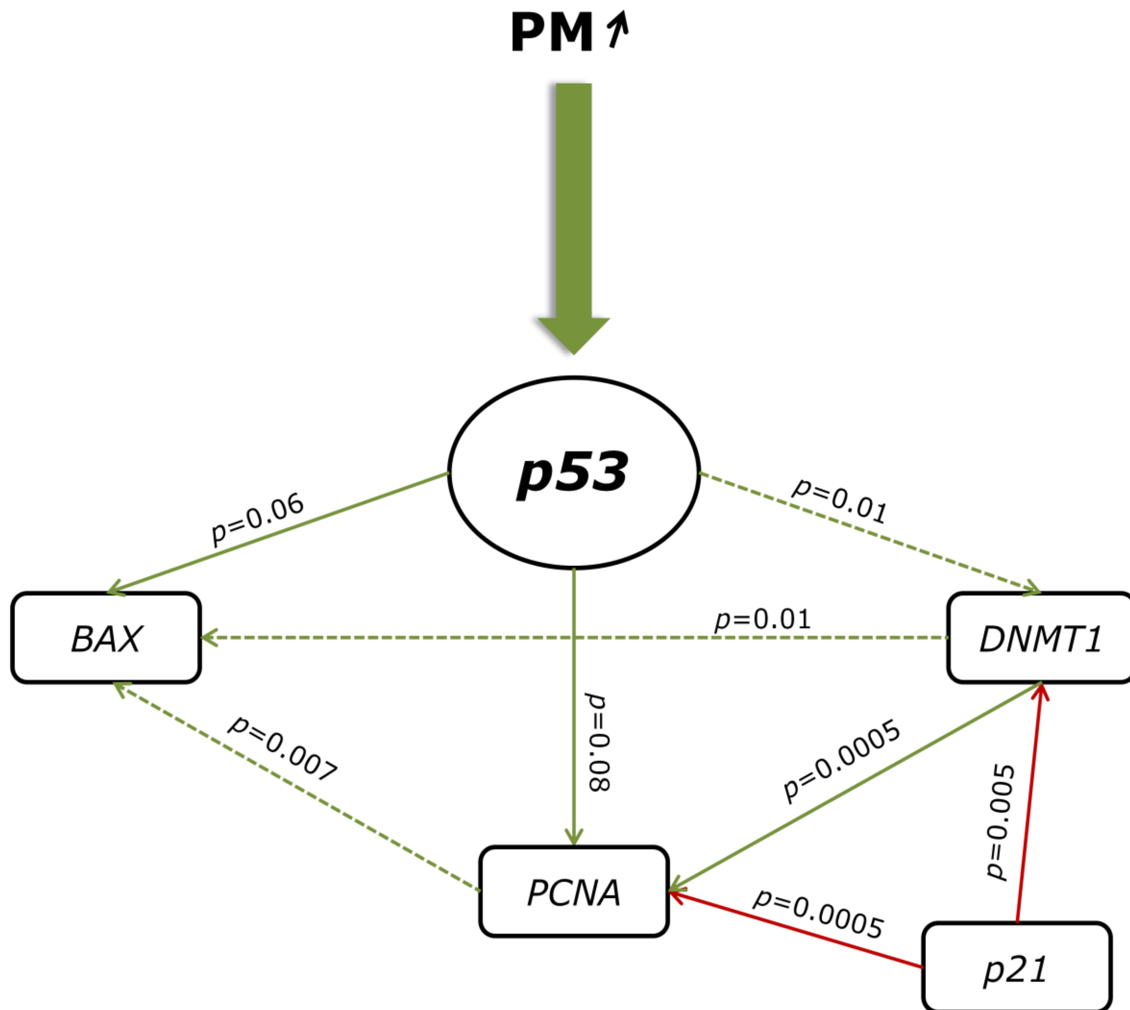

Figure 7: Network based on significant correlation between $PM_{2.5}$ exposure and *p53* gene expression and *p53* and target genes. Direct interactions between genes are shown and include activation (green) and inhibition (red) effects. Dotted lines are associations that were found in this study, but not found or inversed in the micronuclei-formation network published by Van Leeuwen et al. (2011).

4.3 Study limitations

A first limitation of this study is that actual phenotypic MN frequency is not characterized. We can assume MN frequency increases according to PM exposure, but should validate this in future experiments. Although this does not mean we cannot make a conclusion based on gene expression alterations. Forrest et al. revealed that altered expression of specific genes was a potential biomarker of benzene exposure. They did not associate their gene expression with a marker of a genotoxic endpoint either (69).

Although the model was adjusted for several covariates, possibility of residual confounding by some unknown factor that is associated with both *p53* gene expression and ambient air pollution should be taken into account. Another limitation is the limited number of observations and the exposure assessment. Exposures are based on validated models, but only address of residence was taken into account. Address of employment was not incorporated. In addition, ambient exposure does not account for indoor exposure.

4.4 Future perspectives

Future experiments should elucidate the association between gene expression of genes measured in this study and a valid marker of DNA damage. This will give a better insight into using the MN network as a biomarker for MN frequency and thus for chromosomal aberrations or harmful exposures.

Placental tissue is also a valid marker of *in utero* exposure, thus repeating this experiment on placental tissue will give better insights into the effects of PM exposure. It has been shown that responses in placental tissue not always have the same effects as in cord blood. Umbilical cord has a separate circulation that may not be representative of other tissues. Within our research group, Janssen et al. concluded a significant effect of *in utero* PM exposure on mitochondrial DNA content in placental tissue, but not in cord blood (30). Movement of pollutants into the foetal compartments can be blocked or facilitated by placental transporters. It is expected for pollutants to have a bigger influence on placental tissue, since not all pollutants might be able to cross the placenta.

4.5 Practical aspects of gene expression analysis

In order to perform gene expression analysis, RNA quality in cord blood had to be sufficiently high and proper housekeeping genes for normalization of gene expression data had to be determined.

4.5.1 RNA quality in cord blood

RNA will rapidly degrade after blood collection, because of its instability. Therefore, RNA quality and quantity in cord blood was investigated to ensure this was sufficiently high to use for further gene expression analysis. RNA extracted from cord blood collected in PAXgene Blood RNA system and Tempus Blood RNA system, two different blood RNA collection tube systems which stabilize RNA in a different manner, were compared. It has been shown that collection using RNA stabilizing methods gives an increase in RNA stability and yield compared to non-RNA stabilizing collection methods. It is very important to stabilize RNA during sample collection, transport and storage to make sure reliable and reproducible gene expression results are obtained (70). Results showed that RNA purity extracted from cord blood collected in Tempus tubes was by average higher than this of PAXgene tubes, which corresponds with a previous study published by Duale and colleagues (71). Average yield was higher in samples collected in Tempus tubes, and sufficiently high to use the samples in further gene expression analysis. Difference in yield can be explained by a different way of storage after collection. RIN (RNA integrity number) value of RNA extracted from cord blood collected in PAXgene tubes was consistently higher than RNA of cord blood samples collected in Tempus tubes. Samples with RIN value higher than 6 were indicated as good quality and used in further gene expression analysis.

Tempus and PAXgene tubes were compared and our observations showed that both systems gave sufficient RNA quality for further gene expression analysis. We chose to use Tempus RNA blood collection system, on account of practical and financial reasons.

4.5.2 Normalization of gene expression

The first step in normalization of gene expression is the determination of appropriate housekeeping genes. Housekeeping genes are transcribed constitutively in the tissue and it is assumed their expression is not affected by experimental conditions. Normalization by housekeeping genes is necessary to ensure validity of gene expression when measuring mRNA expression with qPCR. This assures no interference of

variables such as the amount of starting material, enzymatic efficiencies, and the differences between tissues or cells in overall transcriptional activity. In general, no difference in efficiency was observed when using cord blood collected in Tempus blood RNA or PAXgene blood RNA tubes.

IPO8, *TBP* and *CYC1* were chosen for normalization of gene expression in cord blood. These genes are not co-regulated and are active in different pathways in the cell. *IPO8* is involved in nuclear import of proteins in the cell. It encodes for a protein which is a member of approximately 20 potential Ran targets that share a sequence motif related to the Ran-binding site of importin-beta. The importin-alpha/beta complex mediates nuclear import of proteins with a classical nuclear localization signal (72). *TBP* or the TATA-binding protein is a general transcription factor that binds to a DNA sequence called the TATA box. *TBP* is a subunit of the eukaryotic transcription factor TFIID, which in turn is part of the RNA polymerase II pre-initiation complex. Binding of TFIID to the TATA box in the promoter region of the gene initiates the recruitment of other factors required for RNA Polymerase II to begin transcription . *CYC1* encodes for a subunit of the cytochrome bc1 complex, which plays an important role in the mitochondrial respiratory chain by transferring electrons from the Rieske iron-sulphur protein to cytochrome c.

5. Conclusion

Research on alterations in gene expression linked to environmental exposures and formation of diseases has been conducted frequently within the last decade. This research is necessary to understand the consequences of environmental air pollution and shows that efforts to minimize environmental pollution are necessary. If environmental pollution is decreased, this will give dramatic improvements in human health, and subsequently reductions in medical costs.

The current study showed a significant association between *in utero* $PM_{2.5}$ exposure during the first trimester of pregnancy and increase in gene expression of tumour suppressor gene *p53*. Increase of *p53* gene expression was associated with activation of response genes *BAX*, *PCNA* and *DNMT1*. These genes are involved in a MN formation network created by Van Leeuwen et al. We observed no significant association between $PM_{2.5}$ exposure and gene expression of *p53* target genes individually, nor in the combined network. However, a positive trend during trimester 1 was observed. It can be concluded that the MN network is affected by $PM_{2.5}$ exposure indirectly by alteration of the central hub *p53* in cord blood. Within this study, we were not able to measure MN formation phenotypes which should be considered in future experiments.

Environmental pollution is a potential risk for human health, and detection of MN in cord blood lymphocytes is an important method for identification of transplacental pollutants in evaluation and protection of children's health. It is well established as a standard method for evaluation of both genomic instability and genotoxic exposure in human biomonitoring studies (73). Biomarkers of exposure are useful in disease epidemiology to understand underlying mechanisms and to characterize high-risk groups. They can also be helpful with improvement of exposure assessment and allow the investigation of individual susceptibility. Response of cells to environmental stress or exposure involves altered gene expression, which is why biomarkers based on gene expression analysis might be a promising new tool for evaluation of exposures. Our findings are a first step into transcriptomic analyses of MN-related genes in response to environmental PM exposure in newborns. Research on genes involved in MN formation and exposure to chemicals, will make it possible to eventually develop a reliable reporter gene assay to screen chemically exposed human populations faster and in a less labour intensive manner than by using microarrays. Although more confirming research is necessary, transcriptomic analysis represents a promising biomarker in epidemiology to estimate exposures and health effects of environmental contaminants.

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7. Supplemental Information

A. Pilot study: RNA integrity in placental tissue

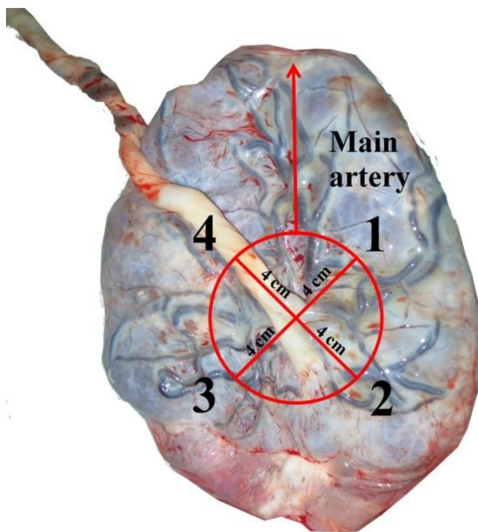

Figure 8: Foetal side of placenta with four standard sites for biopsies relative to the umbilical cord and main artery.

Intracellular RNA is less stable and quality will degrade more quickly than DNA. In a pilot study, RNA integrity of placental biopsies taken on four different time points after delivery was checked. Placentas were obtained in the delivery room and umbilical cord was removed whereupon biopsies were taken immediately. On the foetal side, biopsies were taken on four standard sites relative to the umbilical cord. The first biopsy was taken to the right of the main artery and the three other biopsies in the remaining quadrants of the placenta (Figure 8). The chorioamniotic membrane was removed before the tissue was collected. On the maternal side, one biopsy was taken at the same level of biopsy one on the foetal side. These different biopsies of one placenta should correct for variability within the placenta. Tissue samples were put immediately in RNAlater, a RNA stabilizing reagent, and frozen in liquid nitrogen, whereupon stored in -80°C for long-time storage Subsequently, placentas were stored at -4°C. Biopsies were taken again at 24 hours, 48 hours and 72 hours after delivery in the same manner.

RNA of placental tissue was extracted using the RNeasy mini RNA isolation kit (Qiagen, Valencia, CA, USA) following the manufacturers guidelines. This kit uses silica-membrane RNeasy spin columns with a binding capacity of 100 µg RNA. The samples were eluted in 30 µl RNA free water and stored at -80°C. To make sure no traces of DNA were left in the samples, an extra Turbo DNase treatment was performed on placental tissue with TURBO DNA-free Kit (Ambion, Life Technologies) according to the manufacturers guidelines. Analysis with bioanalyzer gave an RNA integrity number (RIN) for all the samples, of every time point. Only RIN values of RNA extracted from fresh placental tissue were sufficiently high to use RNA in further analyses. RIN values of RNA extracted from placental tissue on time point 24, 48 and 72 were gradually lower. This pilot study showed that only RNA of biopsies collected immediately after delivery was of sufficient quality for further gene expression analysis.

B. Efficiency of reference genes in placental tissue

Table 8: Efficiency of reference genes in placental tissue.

Reference genes	Efficiency (%)
ACTB	91.76
B2M	96.20
CYC1	97.97
HPRT1	89.36
IPO8	102.38
POLR2A	96.67
RPLP0	95.40
TBP	102.64
UBC	102.39
YWHAZ	104.41
GAPDH	93.69

Efficiency within placental tissue were obtained in the same way as in cord blood. Efficiencies are between the range of 90 and 110%.

C. Genorm analysis with placental samples

Determination of appropriate reference genes for normalization of gene expression data in placental tissue was performed in the same way as for cord blood. M-value of *YWHAZ*, *CYC1*, *GAPDH* and *HPRT1* are under the cut-off value of 0.5, thus stable reference genes. V-value shows that use of 2 or 3 reference gens is sufficient for normalization of gene expression.

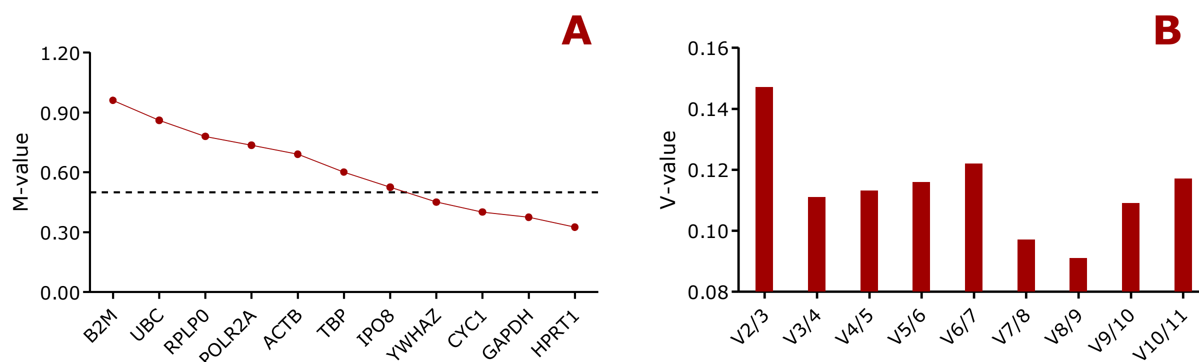

Figure 9: Selection of the most suitable reference genes for normalization in fresh placental tissue using Genorm analysis. The expression stability of candidate genes in placental tissue was determined. A: Stepwise exclusion of the least stable genes by calculating the average expression stability measure M. M-value was calculated for each gene and the least stable gene with the highest M-value was automatically excluded from the next calculation round. The x-axis indicates the ranking of the genes according to their expression stability. B: Determination of the optimal number of reference gens for normalization. X-axis shows the pair-wise variation V_n/V_{n+1} between normalization factors of n and n+1 genes.

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