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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN

master in de biomedische wetenschappen

Masterproef

In utero $PM_{2.5}$ exposure in association with altered gene expression within the miR-210 network

Promotor :
Prof. dr. Tim NAWROT

Copromotor :
dr. Karen VRIJENS

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Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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My love for science has meant that I have chosen for biomedical sciences. During my senior practical training, I had the opportunity to participate in a birth cohort which has made an enormous contribution to my knowledge about environment interactions and human health. This thesis project for my graduation in biomedical sciences was an outstanding opportunity to develop myself in scientific research as in social skills.

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List of Abbreviations

AQP9: *aquaporin-9*

BDNF: *Brain-Derived Neurotrophic Factor*

CI: *Confidence Interval*

COX10: *cytochrome c oxidase assembly homolog 10*

CYP1A1: *cytochrome P450 1A1*

CYP1B1: *cytochrome P450 1B1*

E: *Estimate*

E2F3: *E2F transcription factor*

ENPP2: *ectonucleotide pyrophosphatase 2*

ENVIR*ON*AGE: *ENVIRonmental influence ON AGEing*

ETC: *Electron Transport Chain*

FGFRL1: *fibroblast growth factor receptor-like 1*

GGT: *gamma-glutamyl transferase*

HIF1-α: *Hypoxia inducible factor 1-α*

HRE: *Hypoxia Responsive Element*

HOXA1: *Homeobox A1*

IRC: *Inter-run calibrators*

ISCU: *Iron-sulfur (Fe-S) cluster scaffold proteins*

miRNA: *microRNA*

NO_2: *Nitrogen dioxide*

NTC: *non-template controls*

PM: *Particulate Matter*

$PM_{2.5}$: *Particulate Matter with an aerodynamic diameter $\leq$ 2.5*

PM_{10}: *Particulate Matter with an aerodynamic diameter $\leq$ 10*

PTPN1: *protein tyrosine phosphatase non-receptor type 1*

qPCR: *Real-time quantitative PCR*

ROS: *Reactive Oxygen Species*

SYP1: *Synapsin 1*

TP53I11: *tumor protein p53-inducible protein 11*

WHO: *World Health Organization*

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Abstract

Background: Studies have shown that in utero air pollution can affect the placenta and fetal health. The mechanism how air pollution affects fetal health is not clear. Previous measurement of miR-210 gene expression showed negative association with air pollution. In this study, 5 genes involved in the miR-210 network were selected for gene expression measurements. Investigating possible associations between in utero air pollution exposure and alterations in gene expression of miR-210 network members can reveal if air pollution exerts its effects by targeting the miR-210 network.

Hypothesis: It is hypothesized that in utero air pollution exposure is associated with alterations in the placental expression of genes involved in the miR-210 network.

Methods: Placental biopsies were obtained from 237 mother-newborn participants in the ENVIR*ON*AGE birth cohort and gene expression of 5 selected genes (HIF1- α, PTPN1, COX10, ISCU and RAD52) was determined by real-time polymerase chain reaction. The in utero exposure to different air pollutants (PM_{10}, $PM_{2.5}$ and NO_2) was assessed for trimester 1, 2 and 3 and the entire pregnancy.

Results: The most significant effects found were related to $PM_{2.5}$ exposure. Three genes involved in the miR-210 network showed significant results in association with $PM_{2.5}$ exposure in the adjusted model for cofounders. HIF1-α showed a significant increase in association with $PM_{2.5}$ during the first trimester (estimate (E):1.14; confidence interval (CI): 1.02-1.27), the third trimester (E:1.17; CI: 1.07-1.28), and the entire pregnancy (E:1.42; CI: 1.19-1.70) for each increase of 5µg/m^3 in $PM_{2.5}$. PTPN1 showed a significant increase in association with $PM_{2.5}$ exposure during the third (E: 1.13; CI: 1.04-1.23) trimester and the entire pregnancy (E:1.22; CI: 1.04-1.45) for each increase of 5µg/m^3 in $PM_{2.5}$. Lastly, COX10 showed a significant decrease in association during the first trimester (E: 0.92; CI: 0.87-0.98) for each increase of 5µg/m^3 in $PM_{2.5}$.

Discussion & Conclusion: Perturbations in placental expression of three genes (HIF1-α, PTPN and COX10) involved in the miR-210 network were investigated with increasing in utero $PM_{2.5}$ exposure. HIF1-α is known as the activator of miR-210 and was upregulated in association with exposure and COX10 is a miR-210 target which was down regulated. However, miR-210 showed a down regulation meaning that other genes may play a role in the regulation of gene expression. It is reported that the inflammatory cytokine TNF-α has also an influence on the regulation of gene expression of HIF1-α, PTPN and COX10. It might be possible that air pollution alters the expression of these genes by inducing TNF-α expression. Taken together, the results of this study indicate that in utero PM2.5 exposure can influence the gene expression within the miR-210 network. This provides more insight into the mechanism of air pollution on fetal health.

Samenvatting

Studies hebben aangetoond dat de blootstelling aan luchtvervuiling de placenta en foetale gezondheid tijdens de zwangerschap kan beïnvloeden. Expressie van het miR-210 gen werd binnen deze studiegroep vooraf gemeten en resultaten toonden aan dat miR-210 expressie in associatie met luchtvervuiling gereduceerd was. In deze studie werden vijf genen geselecteerd die betrokken zijn in het miR-210 netwerk. Mogelijke verbanden tussen de blootstelling aan luchtvervuiling tijdens zwangerschap en de veranderingen in genexpressie binnen in het miR-210 netwerk kunnen verklaren of luchtvervuiling effect heeft door het miR-210 netwerk te targeten. De hypothese is dat blootstelling aan luchtvervuiling tijdens de zwangerschap geassocieerd is met veranderingen in placentale genexpressie van genen die betrokken zijn in het miR-210 netwerk.

Voor deze studie werden 237 Placentale biopten verkregen van de deelnemers uit het ENIVR*ON*AGE geboorte cohort. De expressie van de vijf geselecteerde genen (HIF1- α, PTPN1, COX10, ISCU en RAD52) werd opgemeten met real-time polymerase chain reaction. De blootstelling aan verschillende luchtvervuilers (PM_{10}, $PM_{2.5}$ and NO_2) werden bepaald tijdens het eerste, tweede en derde trimester en tijdens de volledige zwangerschap. De meeste significante effecten werden waargenomen gedurende de $PM_{2.5}$ blootstelling. Drie genen die vertoonden een significant resultaat in het gecorrigeerde model voor cofounders. Voor elke stijging van 5µg/m^3 in $PM_{2.5}$, toonde HIF1-α een significante stijging in associatie met $PM_{2.5}$ blootstelling tijdens het eerste trimester (estimate (E):1.14; betrouwbaarheidsinterval (BI): 1.02-1.27), derde trimester (E:1.17; BI: 1.07-1.28) en tijdens de volledige zwangerschap (E:1.42; BI: 1.19-1.70). Ook toonde PTPN1 een significante stijging in associatie met $PM_{2.5}$ blootstelling tijdens het derde trimester (E: 1.13; BI: 1.04-1.23) en de volledige zwangerschap (E:1.22; BI: 1.04-1.45). Vervolgens toonde COX10 een significante daling in associatie met $PM_{2.5}$ blootstelling tijdens het eerste trimester (E: 0.92; BI: 0.87-0.98).

In deze studie werd er aangetoond dat er een verandering is in de placentale genexpressie van drie genen (HIF1-α, PTPN and COX10) die tot het miR-210 netwerk behoren. HIF1-α, de activator van miR-210, toonde een significante stijging wat zou moeten leiden tot een stijging in miR-210 expressie. In tegenstelling tot HIF1-α toonde miR-210 een daling evenals zijn target COX10. Er werd aangetoond dat de expressie van HIF1-α, PTPN en COX10 gereguleerd kan worden door inflammatoire cytokines zoals TNF-α. Dit kan erop wijzen dat luchtvervuiling de genexpressie kan veranderen door de productie van TNF-α te induceren. De bekomen resultaten van deze studie tonen aan dat blootstelling van $PM_{2.5}$ tijdens de zwangerschap de genexpressie kan beïnvloeden binnen in het miR-210 netwerk. Dit zal meer inzicht bieden in de mechanismen van luchtvervuiling op de foetale gezondheid.

1.Introduction

1.1 Air pollution

Air pollution exposure is an important health problem worldwide, because of its role in the development of several diseases [1]. It consists of a complex mixture of gaseous pollutants such as nitrogen dioxide (NO_2) and small particles referred to as particulate matter (PM). PM is a parameter for air pollution which is used in scientific studies to measure air pollution. It is a complex mixture of small particles that vary in size and composition. These particles are released by different sources such as motor vehicles, factories, power plants, windblown dust etc. and enter the human body through the respiratory, gastrointestinal and dermatological route. Particles smaller than 2.5 µm are called $PM_{2.5}$ and particles smaller than 10 µm are called PM_{10} (figure 1). Importantly, the size is determinative for the deposition site in the respiratory tract. PM_{10} particles accumulate mainly in the upper respiratory tract while $PM_{2.5}$ particles can penetrate within the lung alveoli. Hence, they can access the blood circulation and may have different effects at organ level. Thus, the smaller the particles the more harmful they are in terms of health [2]. The inhalation of these particles can create serious problems at different levels of the human body, in which the period of exposure plays an important role [3].

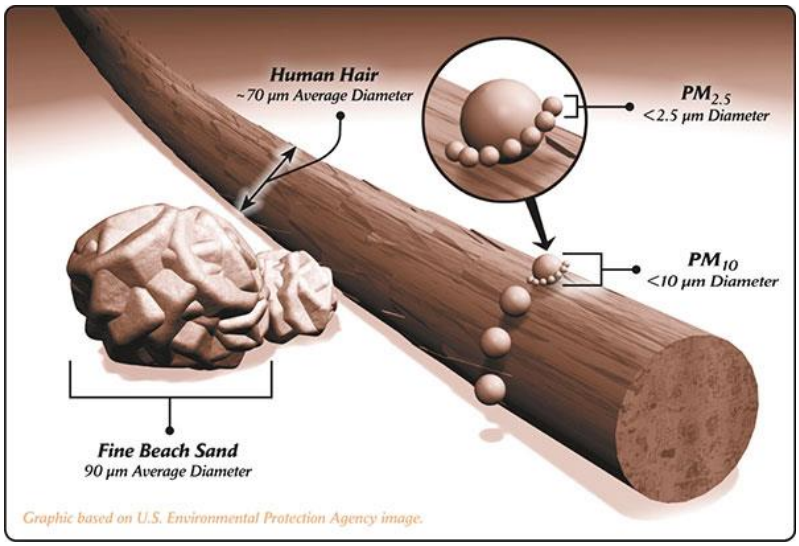

***Figure 1**: A representation of the PM particles* **[4]**.

NO_2 belongs to the gaseous pollutants and is formed by combustion of fossil fuels. Combustion leads to the formation of nitrogen oxides that reacts with ozone or radicals in the atmosphere which

consequently leads to the formation of NO_2. The inhalation of this gaseous NO_2 mainly affects the respiratory system [2].

1.1.1 Molecular mechanisms of air pollution in disease progress

The World Health Organization (WHO) reported that air pollution was estimated to have cause approximately three million premature deaths worldwide in 2012 [5]. Scientists discovered that there is an association between air pollution exposure and cardiovascular and respiratory morbidity and mortality [6]. Children and the fetus are more susceptible to the effects of air pollution. This susceptibility can be explained by their immature immune system, brain and lungs at birth, organs which continue to develop until the age of 6 [7].

The respiratory system is an important target of air pollutants. PM exposure can lead to deep penetration of the particles within the lungs and induce inflammation. Air pollution is an important risk factor for asthma. Recent research has shown that there is an association between childhood asthma and traffic-related air pollution. The results showed that higher exposure to traffic-related air pollution can lead to increased risk for developing asthma in children [8]. Studies have also shown that air pollution is associated with the development of lung cancer [9] [10]. The respiratory system is an important target for NO_2 exposure as well. Infante-Rivard showed a dose dependent significant association between NO_2 exposure and asthma incidence in preschool children [11].

Besides the respiratory system, air pollution has an effect on other systems as well. Air pollution can induce changes in blood clotting and obstruct blood vessels which can lead to cardiovascular complications. In addition, PM particles can reach the nervous system and can damage its structures. The urinary system can be a target of PM particles, whereby they can induce kidney damage. Taken together, air pollution can have serious health effects in the human body. Hence, air pollution exposure must be checked meticulously [2].

1.1.2 Air pollution exposure in association with changing miRNA expression and gene expression

Gene and environmental interactions play an important role in disease development, whereby microRNAs (miRNAs) function as key regulators of human health and disease. MiRNAs are small endogenous, non-coding, single-stranded RNA molecules that control the gene expression by post-transcriptional regulation and are involved in RNA silencing [12]. Epidemiological studies about miRNAs and air pollution can lead to more insight into the environmental exposure effects in healthy individuals [13]. Several studies have shown alterations in miRNA expressions in response to exposure [14] [15]. Bollati et al. showed an up-regulation of miR-21 in response to PM exposure, which plays an important

role in the cardiovascular system [15]. Changing profiles in miRNA expression in response to environmental exposures also play an important role during fetal development [16]. Recently, Maccani et al. investigated the effect of tobacco smoke on in utero development by looking into miRNA profiles. The results showed a significant down regulation of miR-16, miR-21 and miR-146a in the placenta [17]. Taken together, miRNAs form an important link between air pollution exposure and disease progress [18].

Air pollution can also have effect on gene expression. Gaultieri et al. showed that exposure of A549 cells to $PM_{2.5}$ was associated with the induction of cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1) genes which belong to cytochrome p450 and play a role in drug metabolism [19]. Additionally, Takahashi et al. found that NO_2 exposure can lead to increased gamma-glutamyl transferase (GGT) gene expression in the lungs of mice [20]. Seanen et al. looked at placental gene expression levels in the Brain-Derived Neurotrophic Factor (BDNF) pathway in response to in utero exposure to $PM_{2.5}$. They found a significant decrease of two genes (BDNF and Synapsin 1 (SYN1)) which play an important role in neurodevelopment [21]. Therefore, investigation of gene expression levels within the placenta might be an interesting tool since research has revealed that air pollution can alter gene expression within the placenta.

1.2 Placenta

The placenta plays an important role during fetal development. It serves as a barrier between the maternal and fetal circulation, whereby necessary elements for the fetus are transported from maternal blood through the placenta and waste is transported back to the mother [22]. Therefore, normal placental development is needed for a healthy fetal growth and maintenance of the pregnancy. Structural abnormalities can lead to pregnancy complications [23]. By its function as a nutrient transporter, the placenta is a key regulator of fetal programming. Changes in maternal environment lead to perturbations in placental function that can affect fetal programming [24].

1.2.1 Air pollution exposure effects on the placenta and fetus

Air pollution exposure has an influence on placental and fetal development. It has been shown that high PM exposure during pregnancy is associated with fetal growth restriction, low birth weight and preterm birth [23]. In addition, it can lead to preeclampsia and gestational hypertension [23] [7]. The cause of these maternal and fetal complications can be due to alterations during early placental development. More specifically, high exposure can induce oxidative stress and systemic inflammation which can perturb the placental morphology and function and may lead to placental inflammation [23]. Newborns

with a low birth weight are at higher risk for asthma, hypertension and neurological complications in later life [25]. Taken together, several researches have shown the harmful effects of air pollution on fetal health but the underlying mechanisms are not clear. Therefore, it is important to reveal the mechanisms behind the in utero air pollution effects on the fetus.

Epigenetic programming plays an important role during fetal development. Air pollution can alter epigenetic mechanisms which can consequently lead to a predisposition to several diseases in later life. Alterations in the epigenetic mechanisms can be due to the toxicity of the PM particles and these epigenetic changes can consequently influence gene expression [26].

1.3 Hypoxia

Hypoxia is a condition characterized by a low oxygen supply. A cell is defined hypoxic when the oxygen tension falls beneath a certain threshold. This can be dangerous for the whole body [27]. The induction of hypoxia leads to the up regulation of different genes that play a role in different pathways such as apoptosis, cell proliferation, cell cycle etc. The response to hypoxia is regulated by the transcription factor hypoxia inducible factors (HIFs). Hypoxia plays an important role during the first trimester of the pregnancy to ensure normal placental development. It regulates placental vascularization and trophoblast differentiation [28]. HIF-1 plays a role in trophoblast differentiation and vascularization of the placenta by activation of different genes [29]. MiR-210 is activated by HIF1-α and is upregulated during hypoxia [30].

1.3.1 miR-210

There are several miRNAs identified which are regulated by hypoxia. Many of them are tissue or cell-type-specific and are only reported in a limited number of studies [30] [31]. However, different experiments showed that miR-210 is the only miRNA that is mainly expressed under hypoxic conditions, not only in cancer cells but also in normal cells [27]. MiR-210 expression is mainly regulated by the alpha subunit (HIF1-α and HIF2-α). The expression is induced by binding of the alpha subunit to a hypoxia responsive element (HRE) on the proximal miR-210 promoter. This HRE coordinates the expression of miR-210 downstream genes [30]. It plays a role in apoptosis, angiogenesis, cell cycle, mitochondrial metabolism, immunosuppression and the DNA damage response (figure 2) [27]. Additionally, Muralimanoharan et al. showed an upregulation of miR-210 during preeclampsia and investigated the effects on mitochondrial metabolism. They concluded that miR-210 upregulation during preeclampsia can explain the placental mitochondrial dysfunction [32]. All in all, it would be interesting to investigate the placental expression levels of mir-210 and its target genes in association with air pollution exposure.

This could provide more insight into the question whether air pollution exposure during pregnancy is related to hypoxia.

1.3.2 Targets in miR-210 network

RAD52 in DNA damage response

A cell contains DNA repair mechanisms that detect and repair DNA damage. MiR-210 represses DNA damage response by targeting RAD52. RAD52 is an important gene in DNA repair [33]. It repairs double-stranded DNA breaks by homologous recombination. This is a process whereby nucleotide sequences are exchanged between two identical DNA strands [34]. The inhibition of RAD52 can explain the suppressed DNA repair during hypoxia [27].

ISCU and COX10 in Mitochondrial metabolism

Mitochondrial respiration plays an essential role in the sensing of the cellular oxygen concentration. After the initiation of maternal blood flow to the placenta, the hypoxic condition of the placenta turns to a state of oxidative stress. Mitochondrial respiration plays an important role in the cellular energy generation during abundant oxygen supply. Hereby, cellular energy is generated by the tricarboxylic acid (TCA) cycle that generates 38 moles ATP from glucose [30]. However, hypoxia leads to a metabolic shift, whereby cells switch to glycolysis by suppressing mitochondrial respiration. MiR-210 regulates this metabolic shift by downregulating the mitochondrial electron transport chain (ETC) activity. Iron-sulfur (Fe-S) cluster scaffold proteins (ISCU) are an important target of miR-210. ISCU belongs to a machinery that enables the incorporation of Fe-S clusters into enzymes that function in the TCA cycle as well as mitochondrial ETC complexes (I, II and III). Besides ISCU, cytochrome c oxidase assembly homolog 10 (COX10) is an important target as well. It catalyzes the electron transfer from reduced cytochrome c to oxygen and is also a part of the ETC complex IV [27]. Consequently, the expression of mir-210 must be strictly regulated during the pregnancy to achieve a normal placental development and fetal growth.

PTPN1 in Immunosuppression

A recent study has shown that upregulation of miR-210 in hypoxic areas induces immunosuppression in cancer cells. This function is mediated by 3 target genes: protein tyrosine phosphatase non-receptor type 1 (PTPN1), homeobox A1 (HOXA1) and tumor protein p53-inducible protein 11 (TP53I11) which are involved in the regulation of immune responses [35]. MiR-210 leads to immunosuppression by degrading its downstream target genes. PTPN1 is a negative regulator of inflammatory responses by targeting the cytokine receptors and receptor tyrosine kinases. Additionally, PTPN1 and TP53I11 have been shown to be involved in the regulation of cell survival and apoptosis while HOXA1 is involved in cell proliferation

and tumor initiation. Therefore, degradation of these targets leads to the induction of immunosuppression by miR-210 [36].

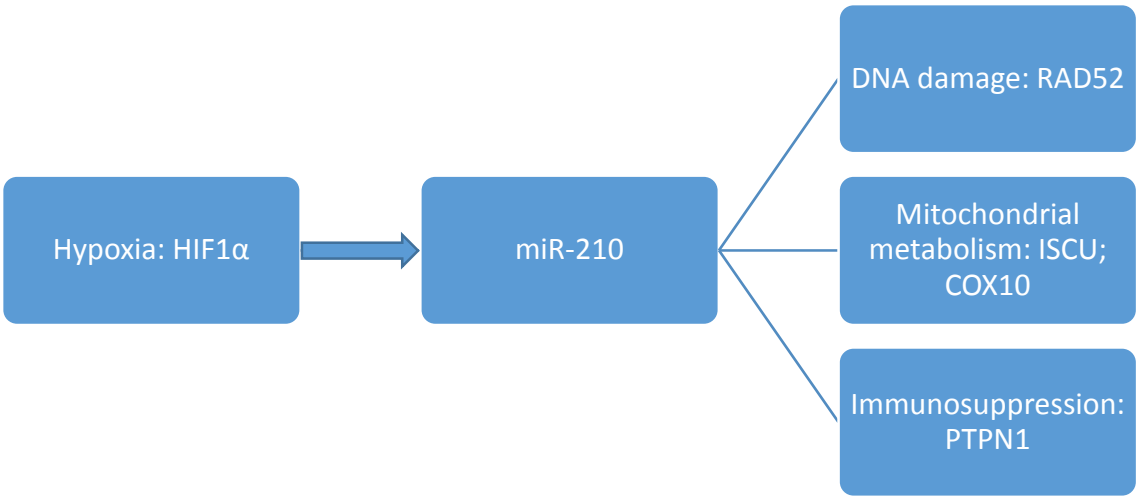

***Figure 2**: Representation of miR-210 and its targets in different mechanisms* **[35]**. *Hypoxia leads to the stabilization of hypoxia inducible factor (HIF1-α) which consequently induces miR-210 expression. MiR-210 has downstream targets in several pathways such as DNA damage, mitochondrial metabolism and immunosuppression. It can prevent DNA repair by inhibiting RAD52, alter mitochondrial metabolism by targeting Iron-sulfur (Fe-S) cluster scaffold proteins (ISCU) and cytochrome c oxidase assembly homolog 10 (COX10) and induce immunosuppression by inhibiting protein tyrosine phosphatase non-receptor type 1 (PTPN1).*

1.4 The Study

This study is embedded in the ENVIR*ON*AGE birth cohort (ENVIRonmental influence ON AGEing) which aims to investigate the effects of air pollution during early life. This cohort consists of about 800 mother-newborn pairs that will be followed into adulthood.

Air pollution exposure during pregnancy has an important influence on placental morphology, function and fetal health. Hypoxia plays an important role during the first stage of placental development. However, the occurrence of hypoxia during the later stages of the pregnancy is dangerous for the fetal development as well as for the mother. MiR-210 is a microRNA which is induced during hypoxia. Previous measurements of miR-210 expression within this cohort showed a significant negative association with in utero $PM_{2.5}$ exposure. This microRNA has several targets in apoptosis, cell proliferation, differentiation etc. Therefore, it is interesting to investigate if air pollution exposure has an influence on miR-210 target genes.

Hypothesis

It is hypothesized that in utero exposure to air pollution is associated with alterations in the expression of genes involved in the miR-210 network. This study aims to investigate the effects of in utero exposure on the placental expression of genes in the miR-210 network. Therefore, the expression of four selected miR-210 targets (RAD52, COX10, ISCU, PTPN1) and activator HIF1-α will be measured within the placenta. The objective of this study is to assess the association between gene expression levels of genes from the miR-210 network and air pollutants. This project will provide more insight into the possible mechanisms underlying the effects of air pollution on fetal development.

2.Materials and Methods

2.1 Study population and sample collection

For this study, 237 placental tissue samples were used from the ongoing ENVIR*ON*AGE birth cohort. The placentas were collected immediately after delivery and snap frozen within 10 minutes at East-Limburg Hospital in Genk. Perinatal parameters such as birth weight, length, date, gestational age, gender and ultrasonic data were retrieved from medical records. Informed consent was provided from the participants and all procedures were approved by the ethical committee of Hasselt University and East-Limburg Hospital in Genk. Only mothers that were able to fill out the questionnaires in Dutch were included. This questionnaire provides detailed information about: mother´s body mass index (BMI), age, education, smoking status, alcohol consumption, occupation, place of residence, medication use, parity and newborn´s ethnicity.

The placentas were frozen at -20°C and were thawed after maximum 48 hours. After thawing, the placental biopsies were taken according to standard procedures reported by Adibi et al. In brief, biopsies of approximately 1 to 2 cm^3 were taken at four standardized sites across the middle region of the fetal side of the placenta. These sites were approximately 4 cm away from the umbilical cord. The first biopsy was taken from the first quadrant to the right of the main artery and the other three from the remaining quadrants (figure 4). Lastly, each biopsy was placed in RNA later for 24 hours at 4°C. Afterwards, the RNA biopsies were stored at -20°C until extraction.

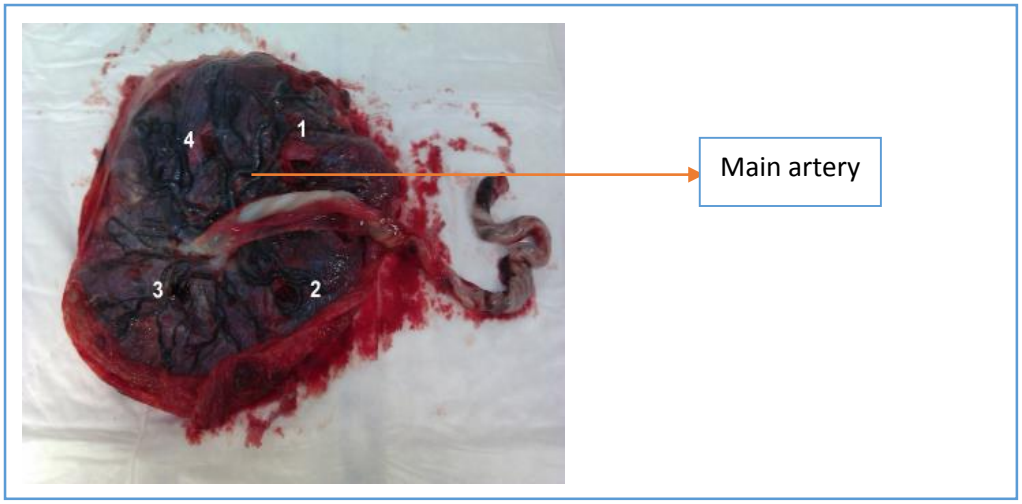

Figure 3*: Placenta biopsies. The numbers correspond with the places were the biopsies are taken. Number 1 is the place where the first biopsy is taken.*

2.2 Exposure estimates

The individual PM_{10}, $PM_{2.5}$ and NO_2 exposures (µg/m³) were calculated for the different trimesters and entire pregnancy. The date of conception was estimated by ultrasound data. The spatial temporal interpolation method (kriging) was used for the calculations in combination with monitoring stations (n=34). This model uses land cover data retrieved from satellite images, based on the residential address of the mother. It also provides interpolated PM_{10}, $PM_{2.5}$ and NO_2 values from the Belgian telemetric air quality networks in 25 x 25 km grids. Mean daily temperatures and relative humidity for the addresses were retrieved from the Royal Meteorological Institute (Brussels, Belgium) [21] [37].

2.3 Total RNA extraction and purification

Before RNA extraction, the placental biopsies (1, 2, 3 and 4) were pooled. RNA extractions were performed with the miRNeasy mini kit (Qiagen, N.V. Venlo, the Netherlands). Two metal homogenization beads and 700 µL QIAzol lysis reagent were added to the RNAlater stabilized placental tissue. Thereafter, the tissue was homogenized, spun down and incubated at room temperature. Subsequently, 140 µL chloroform was added and the samples were incubated at room temperature and centrifuged for 15 minutes at 12000 g at 4°C. After the centrifugation step, the RNA containing aqueous layer was transferred to a new tube and 1.5 volume of ethanol (100%) was added. Afterwards, the samples were transferred into an RNeasy mini spin column and centrifuged at room temperature for 15 seconds at 1000 g. Thereupon, 500 µL RPE buffer was added twice and the samples were centrifuged for 15 seconds and 2 minutes at 10000 g. Finally, the samples were eluted with 30 µL RNAse-free water, incubated at room temperature and centrifuged for 1 minute at 10000 g. The concentration of the samples was measured with a Nanodrop spectrophotometer (ND- 1000; Isogen Life Science, De Meern, The Netherlands) and stored at -80°C. Quality was assessed by determining RIN values using the bioanalyser.

RNA purification was performed for the samples with low purity (OD 260/280 and OD 260/230 < 1.5). Therefore, $1/10^{th}$ of total sample volume 3M Na-acetate (PH5.2) and $7/10^{th}$ of the total volume isopropanol (100%) was added to the samples. The samples were centrifuged for 30 minutes at 13400 rpm at 4°C. 400 µL ice-cold ethanol (70%) was added and the samples were centrifuged for 10 minutes at 13400 rpm at 4°C. An additional centrifugation step of 2 minutes was performed to remove all the supernatant. Finally, the samples were eluted with 40 µL EAE buffer (Magmax) and centrifuged for 30 seconds at 13400 rpm. The concentration of the samples was measured with a Nanodrop spectrophotometer (ND- 1000; Isogen Life Science, De Meern, The Netherlands) and stored at -80°C.

2.4 cDNA synthesis

For the cDNA synthesis, a reaction mix was prepared by using the Goscript reverse transcription system kit (promega, Madison, Wisconsin, USA). The cDNA synthesis was performed for a total concentration of 2000 ng and final volume of 40 µl. First, Oligo dT primer and random primers (0.5µg/reaction) were added. Thereafter, the samples were incubated at 70°C for 5 minutes and cooled on ice for 5 minutes. A reverse transcriptase mix was prepared that contains: nuclease-free water, Goscript 5x Buffer, $MgCl_2$(1.5 mM-5mM), PCR nucleotide mix, Recombinant RNasin Ribonuclease inhibitor and GoScript Reverse transcriptase. Finally, the samples were incubated on a PCR device for: 5 minutes at 25°C, 60 minutes at 42°C and 15 minutes at 70°C. The cDNA samples were stored at -20°C until further measurements.

2.5 Real-time quantitative PCR (qPCR): Gene expression

Real- Time Polymerase Chain Reaction (qPCR) was performed to measure gene expression in placental tissue. For the qPCR measurements, the cDNA samples were diluted to a final concentration of 3.125 µg with nuclease free water and transferred to a 384 well plate. Each sample was measured in triplicate and 2 µl of the dilution sample was added to each well of the qPCR plate together with TaqMan Fast Advanced Master Mix (Life Technologies, Foster City, CA, USA) and PrimeTimeTM assay (Integrated DNA Technologies, Coralville, IA, USA), in a final reaction volume of 10 µL. Three non-template controls (NTC) and six inter-run calibrators (IRC) were taken along in each PCR plate. The NTCs were taken to control for contamination and the IRCs were taken to calibrate between multiple plates. Lastly, the samples were analyzed in a 7900HT Fast Real-Time PCR system (Life Technologies, Foster City, CA, USA) with standard cycling conditions (table 1).

After qPCR, the raw data were collected with the SDS 2.3 software. The Ct values for the selected genes in the miR-210 network were normalized relative to the four reference genes by the qBase software (Biogazelle, Zwijnaarde, Belgium). IPO8, POLR2A, UBC and GAPDH were taken as reference genes as previously determined in the laboratory (data not shown). The qBase program makes it possible to transform the measured Ct values in relative values by taking IRCs and reference genes into account. This corrects for run-to-run differences between multiple plates for each gene.

Table 1: *Standard cycling conditions*

Temperature protocol			
Cycles (#)		Temperature (°C)	Time
1		50	2 minutes
1	**Polymerase activation**	95	10 minutes
40	**Denature**	95	15 seconds
40	**Anneal/Extend**	60	1 minute

2.6 Statistics

The statistical analysis was performed by using SAS software (SAS 9.4, Institute Inc., Cary, NC, USA). The association between miR-210 targets (RAD52, COX10, ISCU, PTPN1), activator HIF1-α, and air pollutants ($PM_{2.5}$, PM_{10} and NO_2) was assessed by linear regression. Each gene was used as a dependent value and air pollution exposure was used as an independent value. Estimated change in gene expression was considered significant for P-values <0.05. The results were adjusted for the following confounders: maternal age, duration of the pregnancy, BMI, newborn's gender, parity, apparent temperature, maternal education and smoking status of the mother. The categorical data were presented as frequencies and numbers, while continuous data were presented as mean and standard deviation.

3.Results

3.1 Study population and exposure characteristics

This study included 237 mother-newborn pairs from the ENVIR*ON*AGE birth cohort (Table 2). The average age for the mothers was 29.3 (± 4.3) and the pregestational BMI was 24.2 (± 4.8) kg/m^2. One hundred and sixty-one mothers confirmed that they had never smoked before, while 39 mothers were past-smokers and 37 mothers were current smokers. A large percentage (55.2%) of the maternal population had a high education. The newborn population consisted of 112 boys. The newborns had a mean gestational age of 39.2 (± 1.3) weeks and a mean weight of 3,435 g (±443.2).

***Table 2**: study population characteristics (n=237)*

Characteristics	Mean ± SD or range and number (%)	Characteristics	Mean ± SD or range and number (%)
Maternal		**Newborn**	
Age, Y	29.3 ± 4.3	Newborn´s gender	
pregestational BMI (kg/m^2)	24.2 ± 4.8	Male	112 (47.3%)
Smoking status		Gestational age (W)	39.2 ± 1.3
Never-smoker	161 (67.9%)	Weight (g)	3,435 ± 443.2
Past-smoker	39 (16.5%)		
Current-smoker	37 (15.6%)		
Parity			
1	120 (50.6%)		
2	94 (39.7%)		
≥3	23 (9.7%)		
Education			
Low	26 (11.2%)		
Middle	78 (33.6%)		
High	128 (55.2%)		
Apparent temperature (°C)			
Trimester 1	9.2 ± 5.5		
Trimester 2	8.7 ± 6.2		
Trimester 3	8.9 ± 5.5		
whole pregnancy	9 ± 2.3		

The average outdoor exposure for the two air pollutants was taken for the different time windows of the pregnancy and the entire pregnancy (Table 3).

***Table 3**: Exposure characteristics*

Pollution indicator	Mean ± SD	25th percentile	75th percentile
PM10 (µg/m3)			
Trimester 1	21.1 ± 5.4	16.7	24.3
Trimester 2	21.2 ± 4.8	17.4	24.1
Trimester 3	21.6 ± 6.1	16.5	25.3
Whole pregnancy	21.2 ± 2.4	19.7	22.7
PM2.5 (µg/m3)			
Trimester 1	16.2 ± 5.3	11.8	20.2
Trimester 2	16.5 ± 5.1	11.9	20.1
Trimester 3	16.6 ± 5.9	11.5	20.8
Whole pregnancy	16.4 ± 2.5	14.7	17.9
NO_2 (µg/m3)			
Trimester 1	20.4 ± 5.9	15.8	25.1
Trimester 2	20.8 ± 6.2	16.1	24.7
Trimester 3	20.7 ± 6.5	16.3	24.5
Whole pregnancy	20.6 ± 4.4	17.7	21.2

3.2 Gene expression analysis

3.2.1 Primer efficiency

Before starting the measurements, primer efficiencies were calculated to ensure if the primers could be used for the measurements and to determine the optimal cDNA input. The primer efficiency was tested for the selected targets (ISCU, COX10, PTNP1 and RAD52) and inducer HIF1-α of miR-210. The primer efficiency was also checked for the aforementioned reference genes. To test primer efficiencies, a sample mix was prepared from 30 samples (5 µL from each) to make 7 serial dilutions from 25 ng/µL for the calculation of the primer efficiency. The primer efficiency was calculated with the formula: $E=10^{(-\frac{1}{a})}-1$. The slope (a) was determined by plotting the mean Ct value from each dilution against the logarithmic dilution concentration. The efficiency is acceptable if it has a value between 90-110%. A value below 90% means that the efficiency is substandard. HIF1-α, RAD52, ISCU, COX10 and PTPN1 showed appropriate results for the primer efficiency (Table 4). Based on the analysis of the efficiency curves, the cDNA was diluted to an optimal concentration of 3.125 ng/µl.

***Table 4**: Primer efficiencies*

Targets	Efficiency (%)
HIF1-α	91%
RAD52	93%
ISCU	95%
COX10	90%
PTPN1	92%
IPO8	92%
POLR2A	97%
UBC	96%
GAPDH	95%

3.2.2 Placental expression of miR210 network genes in association with in utero air pollution exposure

To investigate the association between selected miR-210 network genes and in utero PM10 exposure, linear regression was performed with gene expression data and exposure data for trimester 1, 2, 3 and the entire pregnancy. Three genes from the miR-210 network showed significant associations with PM_{10} exposure during different time windows of the pregnancy. The estimated change in gene expression was calculated for an increase of 5µg/m^3 in PM_{10}. The results were adjusted for maternal education, maternal age, duration of the pregnancy, BMI, newborn's gender, parity, apparent temperature, maternal education and smoking status of the mother. The estimate (E) represents the change in gene expression, whereby E > 1 means an increase and E < 1 means a decrease in gene expression. HIF1-α showed a significant increase in association with PM_{10} exposure during the third trimester (E = 1.14; P = 0.0015) and the entire pregnancy (E = 1.35; P = 0.0007). PTPN1 showed only a significant increase in association with PM_{10} exposure during the third trimester (E = 1.14; P = 0.0064). In contrast to HIF1-α and PTPN1, COX10 showed a significant decrease in gene expression in association with PM_{10} exposure during the first trimester (E = 0.95; P = 0.04). Lastly, ISCU and RAD52 showed no significant changes during the different time windows of the pregnancy (Table 5).

***Table 5**: Estimated change in gene expression in association with PM10 exposure during pregnancy. Values in bold indicate significance in result.*

	HIF1a			PTPN1			*COX 10*		
Time window	**Estimate**	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**
Trimester 1	1.07	0.98, 1.17	0.13	0.98	0.90, 1.07	0.66	0.95	0.90, 1.00	**0.04**
Trimester 2	1.01	0.91, 1.13	0.80	1.01	0.91, 1.13	0.49	1.00	0.94, 1.06	0.94
Trimester 3	1.14	1.05,1.23	**0.0015**	1.14	1.05, 1.23	**0.0064**	1.04	1.00, 1.08	0.11
Whole pregnancy	1.35	1.14, 1.61	**0.0007**	1.36	1.14, 1.61	0.1874	1.00	0.90, 1.11	1.00
	RAD52			ISCU					
	Estimate	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**			
Trimester 1	1.01	0.93, 1.09	0.88	1.03	0.96, 1.11	0.41			
Trimester 2	0.94	0.86, 1.04	0.25	1.01	0.92, 1.10	0.90			
Trimester 3	1.02	0.95, 1.10	0.60	1.02	0.96, 1.09	0.53			
Whole pregnancy	0.98	0.83, 1.16	0.81	1.09	0.94, 1.26	0.23			

***HIF1a**: Hypoxia inducible factor 1; **PTPN1**: protein tyrosine phosphatase non-receptor type 1; **COX10**: cytochrome c oxidase assembly homolog 10; **ISCU**: Iron-sulfur (Fe-S) cluster scaffold proteins.*

To investigate the association between selected miR-210 network genes and in utero NO_2 exposure, the same procedure as for PM_{10} exposure was followed. HIF1-α showed a significant increase in association with NO_2 exposure during the first (E = 1.12; P = 0.0056) and third (E = 1.11; P = 0.0062) trimester and the entire pregnancy (E = 1.12; P = 0.0081). PTPN1 showed a significant increase in association with NO_2 exposure during the first trimester (E = 1.093; P = 0.014), second trimester (E = 1.09; P = 0.008), third trimester (E = 1.095; P = 0.008) and the entire pregnancy (E = 1.12; P =0.0037). COX10, ISCU and RAD52 showed no significant changes in association with NO_2 exposure (Table 6).

***Table 6**: Estimated change in gene expression in association with NO_2 exposure during pregnancy. Values in bold indicate significance in result.*

	HIF1a			PTPN1			COX 10		
Time window	**Estimate**	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**
Trimester 1	1.12	1.03, 1.21	**0.0056**	1.093	1.01, 1.17	**0.014**	0.99	0.95, 1.04	0.8
Trimester 2	1.08	0.99, 1.17	0.073	1.09	1.01, 1.17	**0.021**	1.01	0.97, 1.06	0.59
Trimester 3	1.11	1.03, 1.19	**0.0062**	1.095	1.02, 1.17	**0.008**	0.98	0.94, 1.03	0.43
Whole pregnancy	1.12	1.03, 1.22	**0.0081**	1.12	1.03, 1.21	**0.0037**	1.00	0.95, 1.05	0.92
	RAD52			ISCU					
	Estimate	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**			
Trimester 1	1.03	0.96, 1.11	0.43	0.99	0.93, 1.06	0.87			
Trimester 2	1.02	0.94, 1.10	0.67	0.999	0.93, 1.07	0.98			
Trimester 3	1.04	0.97, 1.12	0.22	0.996	0.94, 1.06	0.90			
Whole pregnancy	1.04	0.96, 1.12	0.36	0.995	0.93, 1.07	0.89			

***HIF1a**: Hypoxia inducible factor 1; **PTPN1**: protein tyrosine phosphatase non-receptor type 1; **COX10**: cytochrome c oxidase assembly homolog 10; **ISCU**: Iron-sulfur (Fe-S) cluster scaffold proteins.*

The association between selected miR-210 network genes and in utero $PM_{2.5}$ exposure was assessed following the same method as outlined above. HIF1-α showed a significant increase in association with $PM_{2.5}$ exposure during the first trimester (E = 1.14; P = 0.021), third trimester (E = 1.17; P = 0.001) and the entire pregnancy (1.42; P = 0.0002). PTPN1 showed a significant increase in association with $PM_{2.5}$ exposure during the third trimester (E = 1.13; P = 0.0041) and the entire pregnancy (E = 1.22; P = 0.0188). COX10 only showed a significant decrease in association with $PM_{2.5}$ exposure during the first trimester (E = 0.98; P = 0.012) (Table 7).

***Table 7**: Estimated change in gene expression in association with $PM_{2.5}$ exposure during pregnancy. Values in bold indicate significance in result.*

	HIF1a			PTPN1			COX10		
Time window	**Estimate**	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**
Trimester 1	1.14	1.02, 1.27	**0.021**	1.03	0.93, 1.14	0.597	0.92	0.87, 0.98	**0.012**
Trimester 2	1.06	0.93, 1.20	0.40	1.02	0.91, 1.14	0.77	1.01	0.94, 1.09	0.68
Trimester 3	1.17	1.07, 1.28	**0.001**	1.13	1.04, 1.23	**0.0041**	1.03	0.98, 1.09	0.23
Whole pregnancy	1.42	1.19, 1.70	**0.0002**	1.22	1.04, 1.45	**0.0188**	0.98	0.88, 1.10	0.77
	RAD52			ISCU					
	Estimate	**95%CI**	**P value**	**Estimate**	**95%CI**	**P value**			
Trimester 1	1.07	0.97, 1.19	0.17	1.01	0.92,1.10	0.89			
Trimester 2	1.02	0.91, 1.15	0.69	0.97	0.88, 1.08	0.62			
Trimester 3	1.03	0.95, 1.12	0.48	1.03	0.96, 1.12	0.39			
Whole pregnancy	1.12	0.95, 1.33	0.18	1.03	0.89, 1.20	0.70			

***HIF1a**: Hypoxia inducible factor 1; **PTPN1**: protein tyrosine phosphatase non-receptor type 1; **COX10**: cytochrome c oxidase assembly homolog 10; **ISCU**: Iron-sulfur (Fe-S) cluster scaffold proteins.*

Since the most significant effects were observed with in utero $PM_{2.5}$ exposure, further analysis were performed with $PM_{2.5}$. Pearson correlation was used to investigate the association between gene expression and in utero $PM_{2.5}$ exposure. Unadjusted analysis showed significant correlations between COX10, HIF1- α and PTPN1 and $PM_{2.5}$ exposure during pregnancy. HIF1-α showed a significant positive correlation for the third trimester ($R = 0.14$; $P< 0.05$) (Figure 4C) and the entire pregnancy ($R = 0.15$; $P< 0.005$) (Figure 4D). In contrast, HIF1-α showed no significant positive correlation for the first trimester and the second trimester (Figure 5A & B). This means that HIF1-α placenta expression was positively correlated with $PM_{2.5}$ exposure during the third trimester and the entire pregnancy.

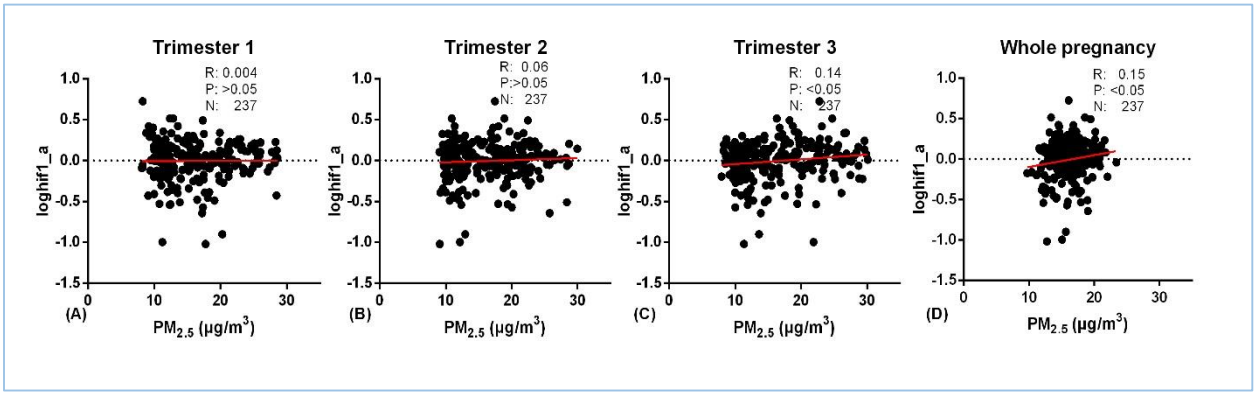

***Figure 4**: Unadjusted correlation between log transformed gene expression of HIF1- α and $PM_{2.5}$ exposure (X = $PM_{2.5}$ exposure during different trimesters and entire pregnancy; Y = log transformed HIF1-α gene expression). The correlation plots indicate a positive correlation with $PM_{2.5}$ exposure during the first trimester (A), second trimester (B), third trimester (C) and the entire pregnancy (D).*

PTPN1 showed a significant positive correlation with $PM_{2.5}$ (µg/m^3) for the third trimester (R = 0.17; P< 0.05) (Figure 5C) and the entire pregnancy (R = 0.15; P< 0.05) (Figure 5D). No significant observations were done for exposure during the first 2 trimesters of the pregnancy.

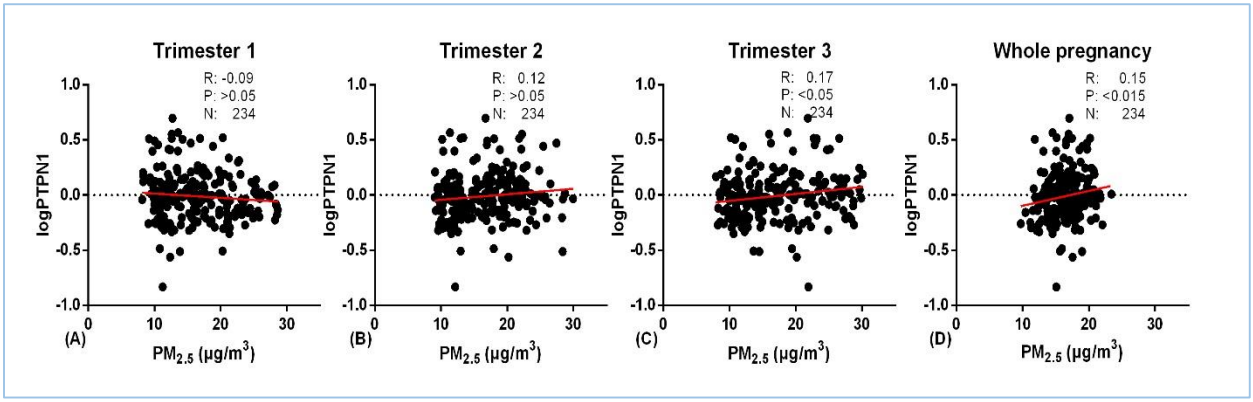

***Figure 5**: Unadjusted correlation between log transformed gene expression of PTPN1 and $PM_{2.5}$ exposure (X = $PM_{2.5}$ exposure during different trimesters and entire pregnancy; Y = log transformed PTPN1 gene expression). The correlation plots indicate a negative correlation with $PM_{2.5}$ exposure during the first trimester (A) and a positive correlation during second trimester (B), third trimester (C) and the entire pregnancy (D).*

COX10 showed a significant negative correlation with $PM_{2.5}$ exposure (µg/m^3) during the first trimester of the pregnancy (R = -0.27; P< 0.05) (Figure 6A). The third trimester showed a significant positive correlation with $PM_{2.5}$ exposure (R = 0.26; P< 0.05) (Figure 6B). No significant positive correlation was observed during the second trimester and the entire pregnancy (Figure 6B & D).

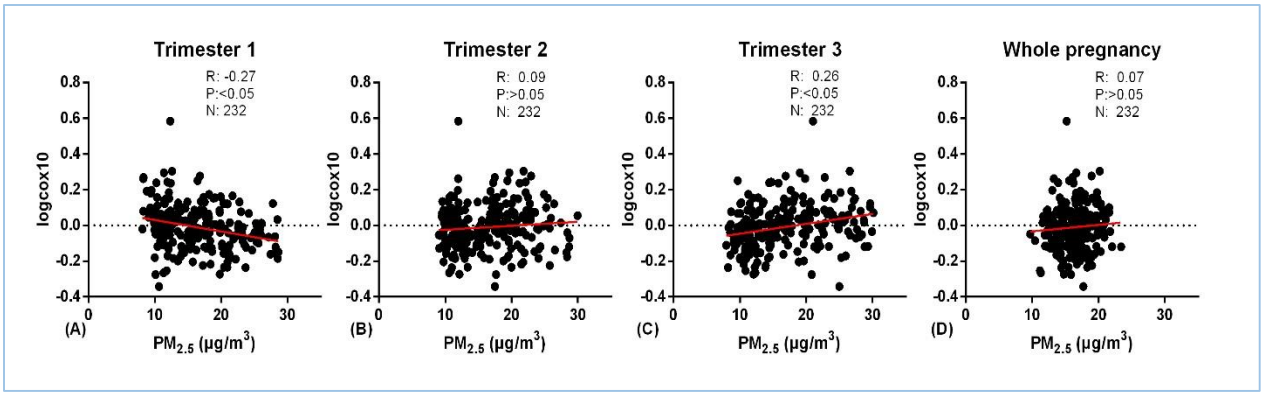

Figure 6*: Unadjusted correlation between log transformed gene expression of COX10 and $PM_{2.5}$ exposure (X = $PM_{2.5}$ exposure during different trimesters and entire pregnancy; Y = log transformed COX10 gene expression). The correlation plots indicate a negative correlation with PM2.5 exposure during first trimester (A) and a positive correlation during the second trimester (B), third trimester (C) and the entire pregnancy (D).*

After adjustment for the aforementioned confounders, HIF1-α gene expression remained positively correlated during the third trimester and entire pregnancy (figure 7). Each increase of 5µg/m^3 in $PM_{2.5}$ was significantly associated with a 16.89% increase in HIF1-α placental gene expression during the third trimester (CI% = 6.65, 28.10; P = 0.001) and of 42.21% during the entire pregnancy (CI% = 18.77, 70.72; P = 0.0002). HIF1-α also showed a significant correlation in trimester 1 after adjustment which was not significant in the unadjusted model. Each increase of 5µg/m^3 in $PM_{2.5}$ was significantly associated with an increase of 13.86% (CI% = 2.07, 27; P = 0.0208) in HIF1-α placental gene expression during the first trimester. PTPN1 gene expression also remained positively correlated with $PM_{2.5}$ exposure during the third trimester and the entire pregnancy. Each increase of 5µg/m^3 in $PM_{2.5}$ was significantly associated with an increase of 13.27% during the third trimester (CI% = 4.12, 23.24; P = 0.0041) and of 22.44% during the entire pregnancy (CI% = 3.55, 44.79; P = 0.0188). COX10 gene expression remained negatively correlated with $PM_{2.5}$ exposure during the first trimester of the pregnancy but not for the second trimester. Each increase of 5µg/m^3 in $PM_{2.5}$ was significantly associated with a decrease of 7.76% in placental COX10 gene expression (CI% = -13.37, -1.80; P = 0.012). Taken together, in utero $PM_{2.5}$ exposure has significant effects on placental gene expression levels for HIF1-α, PTPN1 and COX10 during different time windows of the pregnancy.

Placental miR-210 expression was measured before the start of this project and the data of gene expression was obtained from Drs. Maria Tsamou. MiR-210 showed significant associations with in utero $PM_{2.5}$ exposure during the second trimester and the entire pregnancy. MiR-210 gene expression was significantly decreased with 44.82% for the second trimester (CI: -61.56, -20.78; P = 0.015) and with 53.07% for the entire pregnancy (CI: -72.05, -21.22; P = 0.0047).

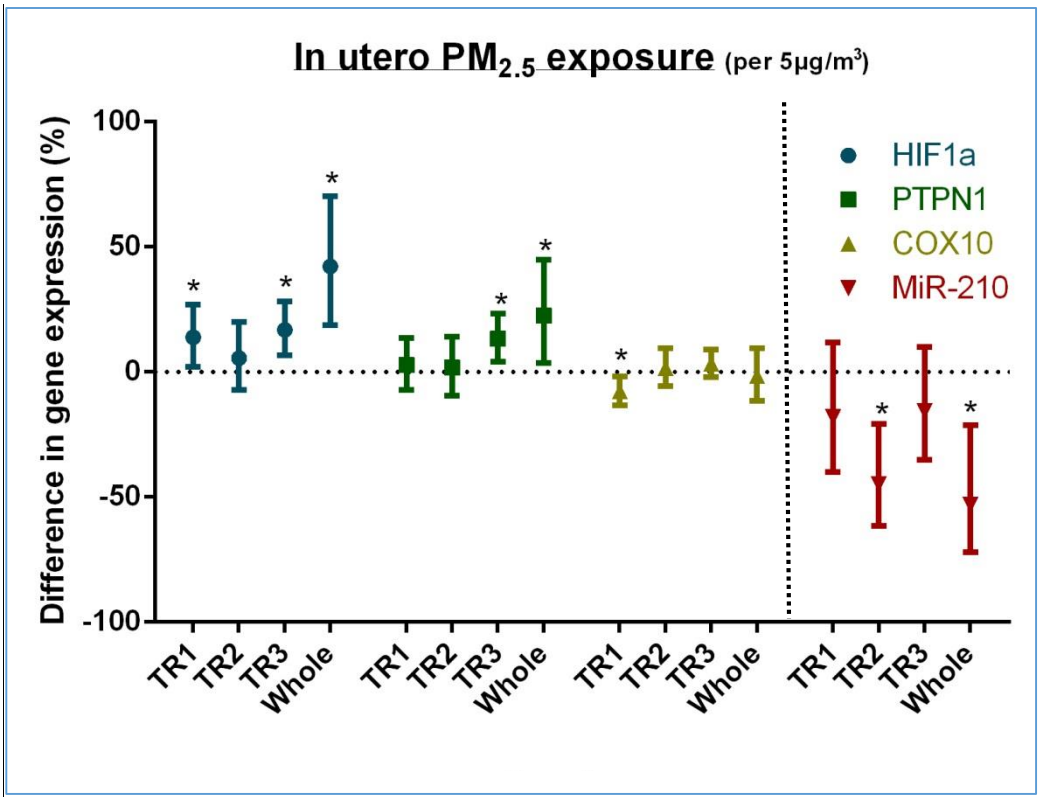

***Figure 7**: Placental expression of HIF1-α, COX10, PTPN1 and mir-210 in association with in utero exposure to $PM_{2.5}$. HIF1- α shows a significant association during the first and third trimester and entire pregnancy,COX10 during the first trimester, PTPN1 during the third trimester and entire pregnancy, while miR-210 shows a significant association during the second trimester and the entire pregnancy. Placental MiR-210 data were obtained by Drs. Maria Tsamou.*

4.Discussion and Conclusion

The main goal of this study was to investigate alterations in the placental gene expression levels for miR-210 network members in association with air pollution exposure during pregnancy. The exposure effects were investigated through different time windows and for the entire pregnancy. The most significant effects were observed with in utero $PM_{2.5}$ exposure. Three genes: HIF1-α, PTPN1 and COX10 from the miR-210 network showed a significant association with $PM_{2.5}$ exposure during different time windows of the pregnancy. Interestingly, miR-210 activator HIF1-α and target PTPN1 showed a significant increase in association with in utero $PM_{2.5}$ exposure, while miR-210 target COX10 showed a significant decrease. MiR-210 gene expression data obtained from previous measurements showed a significant decrease in association with in utero $PM_{2.5}$ exposure. Alterations for these three genes were also observed for in utero PM_{10} exposure. HIF1-α and PTPN1 showed a positive association, while COX10 showed a negative association with in utero PM_{10} exposure. In utero NO_2 exposure showed only a positive association with HIF1-α and PTPN.

These results indicate that there are irregularities within the miR-210 network after PM exposure. It has been reported that miR-210, HIF1-α, PTPN1 and COX10 gene expression can be regulated by other genes. TNF-α is an inflammatory cytokine that can be induced by air pollution. It is shown that TNF-α can lead to increased expression of HIF1-α and PTPN1, while it has an inhibitory effect on COX. It might be possible that air pollution exerts its effect on HIF1-α, PTPN1 and COX10 gene expression by inducing inflammation.

All in all, air pollution can alter gene expression within the miR-210 network. it is presumed that this effect on gene expression may be driven by inflammation. Further investigation is needed to confirm the effect of TNF-α on this genes and elucidate the long-term effects.

In current literature, the association between PM and these three genes was not investigated until now. This is the first study which has investigated the association between air pollution exposure and the expression of genes involved in the miR-210 network.

4.1 Gene expression of the miR-210 network genes in association with in utero $PM_{2.5}$ exposure

4.1.1 HIF1-α

HIF1-α shows a significant increase in association with in utero $PM_{2.5}$ exposure during the first and third trimester and the entire pregnancy. HIF1-α is known as the inducer of miR-210 expression during hypoxic conditions. Therefore, one would expect a significant increase of miR-210 expression. However, MiR-210 gene expression data showed a significant decrease in association with $PM_{2.5}$ exposure during the second trimester and the entire pregnancy. This means that other factors such as inflammation or ROS production besides hypoxia may play a role in the regulation of HIF1-α and miR-210 expression. it is reported that HIF1-α activation not only occurs through hypoxic conditions. J. Patel et al. showed that HIF1-α can be activated during normal oxygen conditions (normoxia) by Immunogenic cytokines and growth factors [38]. ROS plays also a role in the stabilization of HIF1-α not only during hypoxia but also during normoxia [39]. Additionally, miR-210 expression can also be regulated by other genes. Mutharasan et al. showed that miR-210 expression was upregulated after protein kinase B (Akt) overexpression in a mouse model missing the HIF1-α signaling [40]. These findings state that HIF1-α and miR-210 expression can be regulated by other genes which could explain the increased HIF1-α and decreased miR-210 expression in association with $PM_{2.5}$ exposure.

4.1.2 PTPN1

This study showed a significant positive association between PTPN1 and $PM_{2.5}$ exposure during the third trimester and the entire pregnancy. A stronger two fold effect was observed during the entire pregnancy in comparison to trimester 3. The increase in PTPN1 can be due to the significant decrease of miR-210 in association with $PM_{2.5}$ exposure. MiR-210 exerts its immunosuppressive effect by inhibiting PTPN1 activity [35]. It might be possible that air pollution can induce inflammation since PTPN1 plays a role in immune regulation.

4.1.3 COX10

Mitochondrial respiration plays a key role in energy generation during efficient oxygen supply [30]. COX10 is involved in the electron transport chain and its expression must be strictly regulated during pregnancy to ensure normal placental development and fetal growth [27]. Chen et al. reported that miR-210 modulates mitochondrial function by targeting ISCU and COX10 expression under hypoxic conditions which consequently increases ROS production [41]. Therefore, one would expect a decrease in miR-210 expression would lead to increased COX10 expression. However, a significant decrease in COX10 expression was observed in association with $PM_{2.5}$ exposure during the first trimester. This result means

that other genes in the miR-210 network or in other pathways (e.g. mitochondrial respiration, inflammation, apoptosis,…) may play a role in the regulation of COX10 expression.

Different studies have reported that environmental exposure during pregnancy can affect placental gene expression. Seanen et al. found a negative association between in utero $PM_{2.5}$ exposure and BDNF and SYN1 expression [21]. Fei et al. identified a positive correlation between in utero arsenic exposure and placental expression of the aquaporin-9 (AQP9) and ectonucleotide pyrophosphatase 2 (ENPP2) genes. This increase in gene expression was associated with a decreased birth weight [42]. In utero tobacco exposure was associated with an increase of CYP1A1 placental gene expression as well. Suler et al have investigated the placental gene expression of CYP1A1 and observed a significant increase within the placentas of smokers [43]. These studies show that environmental exposure can affect the gene expression within the human placenta. Consistent with these findings, the results of this study showed significant alterations in gene expression for the miR-210 network members in association with in utero $PM_{2.5}$ exposure.

4.2 PM and inflammation

Research has shown that air pollution can induce inflammation. Chuang et al. showed that air pollution is associated with inflammation in humans [44]. Cytokines are signaling molecules and play an important role in the regulation of inflammation and response to harmful agents [45]. Environmental exposure can also increase the expression of inflammatory cytokines within the placenta. Fujimoto et al showed a significant increase of TNF-α and other inflammatory cytokines (IL-2, IL-5, IL-12 α and beta) within mice placenta in association with diesel exhaust exposure [46]. Interestingly, it has been reported that HIF1-α expression can be induced by TNF-α. Tsapourniati et al. reported that TNF-α is capable to increase HIF1-α mRNA transcription under normoxia in airway smooth muscle cells [47]. Beside HIF1-α, PTPN1 expression also can be induced by TNF-α. Zabolotny et al. have shown that TNF-α treatment increases the mRNA levels of PTPN1 in multiple cultured cell types [48]. Lastly, it has also been shown that TNF-α treatment leads to the inhibition of COX (electron transport chain complex IV) activity in different intact cell systems [49]. Consistent with these findings, HIF1-α and PTPN1 showed a significant increase, while COX10 showed a significant decrease in association with PM exposure. Therefore, it might be possible that air pollution can lead to an increased TNF-α production (figure 8). Consequently, TNF-α can activate HIF1-α, increase the PTPN1 expression and decrease COX10 expression. COX10 is a member of COX and it might be possible that decreased COX10 expression in association with PM2.5 exposure is due to decreased COX activity. Thus, it might be possible that TNF-α induction by PM can form the possible link

between air pollution and altered gene expression. Further investigation is needed to confirm this pathway.

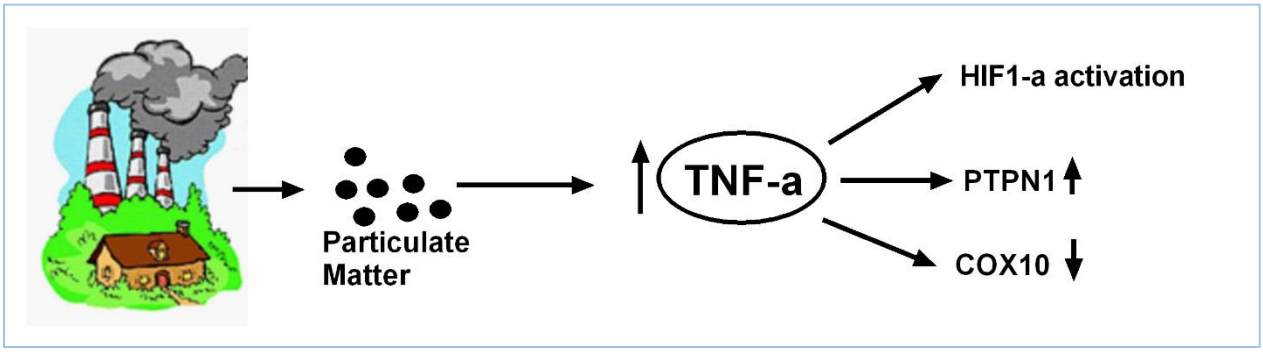

Figure 8: Representation of the suggested pathway how PM leads to alterations in HIF1-a, PTPN1 and COX10 gene expression [45]- [49]*. Particulate matter leads to the induction of TNF-α which consequently leads to an increased HIF1-α and PTPN1 gene expression, while COX10 expression is decreased.*

4.3 Trimester specific sensitivity to $PM_{2.5}$ exposure

Trimester 1: In utero $PM_{2.5}$ exposure during the first trimester of pregnancy showed significant alterations in placental COX10 and HIF1-α expression. The first trimester of the pregnancy is characterized by hypoxic conditions to ensure normal placental development and fetal growth [50]. It might be possible that in utero $PM_{2.5}$ exposure influences the processes in the first trimester. This influence consequently can contribute to altered gene expression.

Trimester 3: In utero $PM_{2.5}$ exposure during the third trimester showed significant alterations in placental PTPN1 and HIF1-α expression. The sensitivity to $PM_{2.5}$ exposure during the third trimester might be explained by the induction of placental inflammation. It might be possible that the third trimester is sensitive for $PM_{2.5}$ induced inflammation which consequently contributes to altered gene expression.

4.4 Difference between in utero PM and NO_2 exposure

In this study, in utero PM_{10} and $PM_{2.5}$ was associated with alterations in gene expression of three genes (HIF1-α, PTPN1 and COX10), while in utero NO_2 exposure was associated with alterations in gene expression of two genes (HIF1-α and PTPN1). The difference between the effect of PM and NO_2 might be explained by the source from which they are formed. The major source for NO_2 is combustion of fossil fuels and motor vehicles [51]. In Europe, the vehicular traffic is tremendously increased and contributes mainly to the formation of NO_2. Additionally, NO_2 can also lead to the formation of other pollutants such as PM [52]. Condensation or chemical reactions that form new particles can lead to nucleation which induces the production of small PM particles [51]. PM particles can be generated from heavy metals that

evaporate during combustion, elemental carbon generated from combustion and organic carbon, sulfates and nitrates [52].

Additionally, Wegmann et al. reported that exposure to high NO_2 concentrations can induce an inflammatory response in mice lungs [53]. Another study in healthy individuals showed that NO_2 exposure can induce airway inflammation [54]. This states that NO_2 can exert its effect by inducing inflammation in the lungs. Consistent with these studies, PTPN1 gene expression was significantly increased in association with NO_2 exposure during all the trimesters and the entire pregnancy. It has been reported that PTPN1 expression plays a role in immune regulation and is induced by inflammation in vivo by TNF-α [48]. Therefore, it might be possible that NO_2 exerts its effect by inducing inflammation at the fetal side of the human placenta.

4.5 European and WHO air pollution standards

The European Union has developed a standard for the mean air pollution exposure for different air pollutants due to the detrimental health effects of air pollution exposure [55]. The mean exposure value of the air pollutants for the entire pregnancy in this study ($PM_{2.5}$: 16.4 µg/m^3; PM_{10}: 21.2 µg/m^3; NO_2: 20.6 µg/m^3) was below the European air pollution standards (Table 8). This demonstrates that even lower exposure levels to air pollution during the pregnancy can affect fetal health. Therefore, further measures should be taken by adapting the European standards to stricter values.

The WHO air pollution standards (table 8) were also taken into account since they are stricter. In comparison to the other pollutants, $PM_{2.5}$ exceeds the WHO standards with a value of approximately 6 µg/m^3. This can explain why the most significant effects were observed with in utero $PM_{2.5}$ exposure.

It is reported that fine particles such as $PM_{2.5}$ are more prominent in impairing human health. Dependent on its size, $PM_{2.5}$ can penetrate more deeply within the lungs and enter the blood circulation [56]. Consequently, it can reach and access different biological compartments in comparison to bigger particles [2]. This can explain why the most significant effects were observed with $PM_{2.5}$ exposure.

***Table 8**: European and WHO air pollution standards* [55] [57]

Air pollutant	Permitted Concentration		Time
	EU	WHO	
PM_{10}	40 µg/m^3	20 µg/m^3	1 Year
$PM_{2.5}$	25 µg/m^3	10 µg/m^3	1 Year
NO_2	40 µg/m^3	40 µg/m^3	1 Year

4.6 Study limitations

The use of placental tissue also has limitations. Sample location, processing time and heterogeneity have an effect on gene expression measurements. The difference in gene expression within the placenta may be due to difference in sample size [58]. To minimize this cofounding effect, the placental biopsies were taken at four standardized sites and pooled.

Another limitation is that gene expression is measured at the end of the pregnancy. Therefore, it is not possible to investigate alterations in gene expression during different pregnancy windows within healthy human placentas. An alternative way to investigate the altered gene expression within a specific trimester can be accomplished by animal studies.

4.7 Future perspectives

Further experiments should be performed to investigate the association between TNF-α expression and air pollution and its effect on HIF1-α, PTPN1 and COX10 gene expression. Therefore, TNF-α expression can be measured within human placenta to investigate the correlation between air pollution. Subsequently, correlation between TNF-α and HIF1-α, PTPN1 and COX10 can be assessed to confirm the aforementioned pathway.

The effects of changed gene expression levels in later life could be assessed in the follow up study of the ENVIR*ON*AGE birth cohort. Newborns that participate within this study are re-invited at an age of four. During the invitation, a picture of the retina is taken to assess the blood vessels in the eye and an echo is performed of the carotid. This will give a first indication about the cardiovascular complications that can occur. Statistics could be performed with this data to investigate if the alterations in MiR-210 network are associated with cardiovascular complications.

Lastly, DNA methylation plays an important role in the regulation of gene expression and embryonic development. DNA methylation within a specific gene switches this gene into an off status [59]. In recent literature, the interest for perturbations in methylation patterns within specific genes assigned to environmental exposure is increasing. Sutler et al. have demonstrated that increased CYP1A1 expression with in utero tobacco exposure was associated with hypomethylation in CYP1A1 promotor region [43]. A previous study from the ENVIR*ON*AGE birth cohort published that placental global DNA methylation was decreased in association with air pollution exposure during early pregnancy [6]. It might be interesting to measure the methylation patterns of the miR-210 network members which showed a significant association with in utero $PM_{2.5}$ exposure. Investigating the methylation patterns can reveal if the

association between in utero $PM_{2.5}$ exposure and altered gene expression within the miR-210 network is mediated by epigenetic modifications.

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