FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef
Prenatal ambient air pollution and cord blood insulin-like growth factor (IGF) in the ENVIR*ON*AGE birth cohort

Promotor :
Prof. dr. Tim NAWROT

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.

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

universiteit hasselt

KNOWLEDGE IN ACTION

Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt
Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek

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Content

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List of abbreviations

ADHD	Attention deficit hyperactivity disorder
BC	Black carbon
DHWS	Dutch Hunger Winter study
ELISA	Enzyme-linked immunosorbent assay
ENVIR*ON*AGE	ENVIRonmental influence ON early AGEing
IGF(2)	Insulin growth factor (2)
IQR	Interquartile range
LOI	Loss of imprinting
PCC	Pearson correlation coefficient
$PM_{0.1}$	Ultrafine particulate matter with a diameter of less than 0.1 μm
PM_{10}	Particulate matter with a diameter of less than 10 μm
$PM_{2.5}$	Particulate matter with a diameter of less than 2.5 μm
SD	Standard deviation
WHO	World Health Organization

Abstract

Introduction

Ambient air pollution is a global public health concern. The Barker hypothesis illustrates the impact of exposures during pregnancy on the child's susceptibility to diseases later in life. Due to a heightened sensitivity during the prenatal period, air pollution may influence fetal development leading to a higher risk of low birth weight and preterm birth. Insulin-like growth factor 2 (IGF2) is an important regulator of placental and fetal growth. In this study, the association between IGF2 expression in cord blood and exposure to particles with a diameter ≤ 10 μm (PM_{10}), ≤ 2.5 μm ($PM_{2.5}$) and black carbon (BC) has been investigated.

Material and methods

Cord blood was collected from 516 mother-newborn pairs recruited from the ENVIR*ON*AGE (ENVIRonmental influence ON early AGEing) birth cohort. The expression of IGF2 was measured using a sandwich enzyme-linked immunosorbent assay. Air pollution levels were determined using a spatiotemporal interpolation method, based on the parents' home address. Statistical analyses were carried out using multiple linear regression models. *A priori* chosen covariates that were taken into account included age, education and smoking status of the mother, sex and ethnicity of the newborn, gestational duration and season at delivery.

Results

For an interquartile range (IQR) increment of 0.35 μg/m³ in annual maternal BC exposure, the IGF2 expression in cord blood decreased with 55 ng/mL (95% CI: -81.91 to -28.15). Corresponding results were found for annual $PM_{2.5}$ exposure (IQR: 2.35 μg/m³) and annual PM_{10} exposure (IQR: 3.51 μg/m³), i.e., -27.8 ng/mL (95% CI: -53.64 to -1.96) and -35.8 ng/mL (95% CI: -64.22 to -7.42) in IGF2 expression, respectively.

Discussion and conclusions

IGF2 protein expression in cord blood was negatively associated with annual air pollution exposure. The potential health effects of air pollution-induced lower IGF2 concentrations at birth are still unclear and require further research.

Samenvatting

Inleiding

Luchtvervuiling vormt een gezondheidsrisico voor de gehele wereldpopulatie. De Barker-hypothese illustreert de invloed van intra-uteriene blootstellingen op de vatbaarheid voor ziektes later in het leven. Door een verhoogde gevoeligheid tijdens de prenatale periode is het mogelijk dat luchtvervuiling de foetale ontwikkeling verstoort en zo een groter risico op vroeggeboorte of een lager geboortegewicht ontstaat. Insuline-achtige groeifactor 2 (IGF2) is een belangrijke regulator in de groei van de foetus en de placenta. In deze studie is de associatie tussen IGF2 expressie in navelstrengbloed en prenatale blootstelling aan partikels met een diameter ≤ 10 µm (PM_{10}), ≤ 2.5 µm ($PM_{2.5}$) en roet onderzocht.

Materiaal en methoden

Na de bevalling werd navelstrengbloed verzameld van 516 moeder-pasgeborene koppels gerekruteerd uit het Limburgs Geboortecohort. IGF2 expressie werd gemeten door middel van een enzym-gebonden immunosorbent assay. De concentratie fijn stof werd bepaald met behulp van spatiotemporele interpolatie gebaseerd op het thuisadres van de ouders. Meervoudige lineaire regressie modellen werden toegepast voor de statistische analyses. *A priori* gekozen covariabelen zoals leeftijd, opleiding en rookgedrag van de moeder, geslacht en etniciteit van de pasgeborene, duur van de zwangerschap en seizoen bij de bevalling werden in rekening gebracht.

Resultaten

Voor een stijging in interkwartielafstand (IQR) van 0.35 µg/m³ blootstelling aan roet in het jaar voor de bevalling, daalde de expressie van IGF2 in het navelstrengbloed met 55 ng/mL (95% CI: -81.91 to -28.15). Overeenkomstige resultaten werden gevonden voor jaar blootstelling aan $PM_{2.5}$ (IQR: 2.35 µg/m³) en PM_{10} (IQR: 3.51 µg/m³), -27.8 ng/mL (95% CI: -53.64 to -1.96) and -35.8 ng/mL (95% CI: -64.22 to -7.42) respectievelijk.

Discussie en conclusie

IGF2 eiwitexpressie in navelstrengbloed is negatief gecorreleerd met jaar blootstelling aan fijn stof. De mogelijke gezondheidseffecten van verlaagde IGF2 concentraties bij geboorte geïnduceerd door luchtvervuiling zijn onduidelijk en vereisen verder onderzoek.

1. Introduction

Since the observations in the Meuse Valley in 1930, the possibly lethal effects of air pollution are recognised (1). **Ambient air pollution** is a severe global public health concern. Several studies link air pollution exposure to human morbidity and mortality, mostly associated to cardiovascular, respiratory or carcinogenic outcomes (2-4). The **carcinogenic** properties of outdoor air pollution are recently confirmed by the World Health Organization (WHO) (5).

Ambient air pollution consists of a mixture of gaseous and particulate toxins expelled from multiple anthropogenic and natural sources (3). These element can arise as primary pollutants that are emitted directly into the environment or they can be secondarily formed in the atmosphere by the interaction of primary pollutants with sunlight, moisture or both (3, 6). A large fraction of the anthropogenic production of pollutants consists of the combustion of fossil fuels. Transportation and industries emit carbon monoxide, nitrogen dioxide, ozone, polycyclic aromatic hydrocarbons, volatile organic compounds and **particulate matter** (PM). These elements can be set free by the pumping of gasoline, vehicle exhaust, the resuspension of settled road dust and the activities of industrial facilities (3).

1.1 Origin of particulate matter

The major component of ambient air pollution is PM; a complex mixture of chemicals and solid and liquid particles suspended in air (7). PM particles can be categorised according to their **size** (figure 1); coarse (PM_{10} with a diameter of 10 µm or less), fine ($PM_{2.5}$: diameter of 2.5 µm or less) and ultrafine ($PM_{0.1}$ with a diameter less than 0.1 µm) (3, 8) A smaller diameter makes the particles capable of penetrating the body more deeply leading to a higher capacity to damage internal systems by inducing local and systemic responses. $PM_{2.5}$ particles are able to reach the lung alveoli where they can interfere with the gas-blood exchange or bring metals, organic compounds and other elements into the circulation. $PM_{0.1}$ is also shown to cross the blood-brain-barrier (2, 8).

PM mostly originates from **manmade** sources including industrial emissions and combustion by-products often related to traffic exhaust fumes (7, 8). The fine particles are also set free by smoking cigarettes, cooking and burning wood in fireplaces (7). Natural sources include pollen, spores, bacteria and plant and animal debris (3).
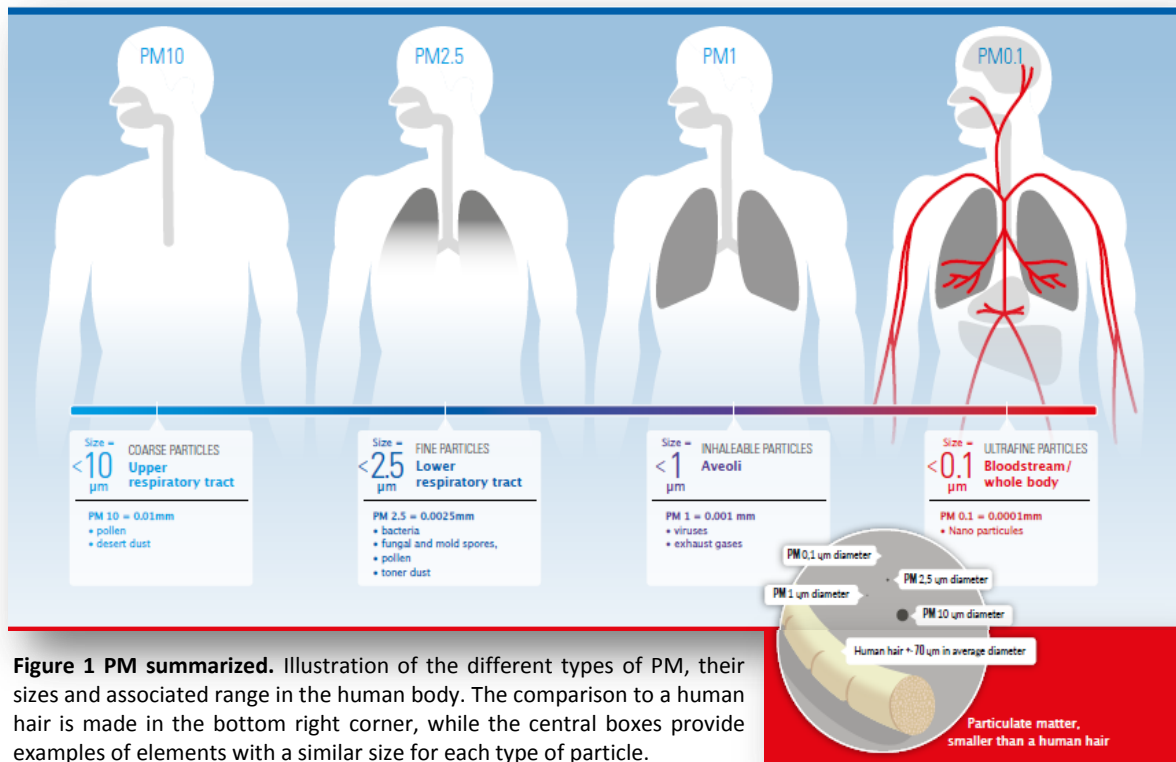
Figure 1 PM summarized. Illustration of the different types of PM, their sizes and associated range in the human body. The comparison to a human hair is made in the bottom right corner, while the central boxes provide examples of elements with a similar size for each type of particle.

1.2 Guidelines for particulate matter exposure

The WHO aims to achieve the highest attainable health for the global population by working together with governments and other partners in more than 150 countries. The **WHO Air Quality Guidelines** are constructed to provide an assessment of health effects and corresponding thresholds for pollution levels harmful to public health. Globally, 92% of the population in 2014 was exposed to air quality that exceeded the determined maximum air pollution concentrations (5).

The WHO Air Quality Guidelines include values for $PM_{2.5}$ and PM_{10} consisting respectively of an annual average of 10 μg/m³ and 20 μg/m³, and a 24-hour mean of 25 μg/m³ and 50 μg/m³. These levels of exposure are not harmless, the average life expectancy is still lowered with 8.6 months in cities where the guideline levels are met. Within the vision of the WHO, the Air Quality Guidelines also provide interim concentration targets to stimulate a gradual decrease in PM levels; if met, the risks for acute and chronic health effects would significantly reduce. The ultimate goal is to progress towards these guideline values (5).

1.3 Health effects of particulate matter

PM shows to be closely linked to an increased bladder and especially lung cancer incidence. Lung diseases are directly correlated to air pollution as inhalation in humans forms the primary source of exposure to pollutants (3). An estimate of 3 million premature deaths per year are caused by exposure to PM_{10} elements: 72% of these are due to heart diseases, 28% originate from respiratory diseases including infections, lung cancer and obstructive pulmonary disease (5).

Damage caused by air pollution exposure before birth may be associated to diseases later in life. In 1990, the concept that prenatal or early life environmental exposures affect health and disease later in life was introduced by David **Barker** (9). A possible mechanism of action is via epi**gen**etic alterations that are shown to persist after the causative factor is removed (3). The basis of this hypothesis consists of anticipatory responses by the fetus or child that permanently influence the cellular and organ differentiation to obtain adaptations that are assumed beneficial later in life. An aberration in the balance between these changes and needs later in life, can form a risk to develop complex diseases (10). Although these factors are not known to induce disease themselves, environmental exposures including air pollution can affect the onset and progress of disease development (11).

Due to the increased **vulnerability** of the lungs and immune system during **childhood**, the prenatal air pollution exposure shows increased effects compared to exposure later in life (12). Children's lungs are still growing and they spend a great deal of time outdoors, often engaging in physical activities that cause an increase in breathing rate that is already higher compared to adults. Lastly, children mostly breath orally meaning that the air does not flow through the nasal filter. All these factors lead to an increased amount of inhaled particles that may alter the lung development and function (3). The possible health consequences include growth retardation leading to an increased risk of low birth weight and preterm birth (13). Later in life, the child can also experience a lowered IQ, airway diseases or neurodevelopment disorders than can be linked to the exposure to pollutants during pregnancy (4, 14).

1.4 Insulin-like growth factors and early-life development

Insulin-like growth factors (IGFs) are named after their significant homology to pro-insulin and identified growth hormone-like effects. IGFs are present in the circulation and can be detected in plasma (15). In the prenatal period, the functions of IGFs consist of regulating **growth**. Maternal IGFs complete their role by actions on both the mother and the placenta. As soon as the placental IGF production starts (around 6 weeks of gestation), the growth and development are also regulated by fetally derived IGFs. These factors only cross the placenta in small quantities, indicating that the role of IGFs produced by the mother is mostly executed indirectly through effects on the maternal metabolism and placental growth leading to a regulation of the nutrient availability for the fetus (16). The effect of IGFs on the placenta comprise of influencing its capacity to supply the fetus of nutrients by regulating placental morphogenesis, nutrient transport and hormone secretion. Hormones can be released into the umbilical circulation to affect the fetal growth, or they can be set free in the maternal circulation to alter the maternal metabolism and substrate availability for placental transfer. After the postnatal growth is complete, the function of IGFs is less clear (16).

IGF2 levels are highest in the fetal circulation and its concentrations rise during pregnancy (15). These levels decline towards the delivery due to the pre-partum maturation of the influenced tissues (16). IGF2 expression is highly active in fetal and placental tissues to regulate cell proliferation, growth, migration, differentiation and survival (15). An elevated maternal IGF2 content during pregnancy causes an increase of the uptake of amino acids by maternal visceral organs and an enhanced fetal growth near term (16). After birth, the IGF2 transcription decreases rapidly in most tissues. The IGF2 expression of the adult is mostly executed in the liver (15).

IGF2 expression is determined by the process of **genomic imprinting**, a type of gene regulation where a specific allele, depending on its parental origin, is silenced by the implementation of DNA methylation marks on its sequence. The IGF2 gene is maternally silenced leading to **paternal expression.** This mechanism is highly important in embryogenesis and placental function. A possible explanation for the antagonistic biologic effects of maternally and paternally imprinted genes is clarified by the **conflict** hypothesis (3). The IGF2 gene is paternally expressed, while the IGF2-receptor that acts as a scavenger for IGF2 is maternally expressed. This mechanism constructs a built-in safety that may neutralize harmful effects caused by abnormal imprinting (15). Despite dubiety concerning the biological relevance, the survival of imprinted genes indicates that its selective advantage outweighs the susceptibility to loss of imprinting (LOI) mutations (15, 17).

The establishment of the imprinted marks occurs during the **epigenetic reprogramming** of the primordial germ cells (figure 2). This reprogramming process is required for normal germ cell development and consists of complete erasure and re-establishment of DNA methylation patterns (18, 19). Specific methylation patterns of particular genomic regions in different cells and tissues are formed resulting in a mechanism for tissue specific gene expression (20). This expression pattern is epigenetically maintained in all adult tissues (21). Epigenetic reprogramming is necessary for the proper monoallelic expression of imprinted genes, to obtain totipotent characteristics of future germ cells and to prevent inheritance of epigenetic defects. The following de novo DNA methylation provides sex-specific methylation of imprinted genes (20, 22).

A second, more extensive round of epigenetic reprogramming involves somatic cells and happens after the fertilisation where a zygote is formed from the egg cell that carries the maternal DNA methylation pattern and the sperm cell carrying the paternal DNA methylation of its producer (19, 20). The paternal epigenome undergoes rapid active genome-wide DNA demethylation while the maternal epigenome undergoes passive, gradual demethylation. Imprinted genes are not involved in this process and will preserve the methylation patterns inherited from the parents. After this, de novo methylation is initiated to re-establish the methylation marks that will be stably inherited in somatic cells of the progeny (20).

Errors in epigenetic reprogramming can lead to transmission of **epimutations** through the germ-line, potentially influencing the fetal development and health in the offspring (19). Aberrations of the fetal growth are linked to an increased risk of morbidity, mortality and of developing degenerative diseases later in life (16). Alterations in the status of imprinted genes can have harmful consequences (17). Due to its role in various processes, disruption of the IGF2 expression is implicated to play a significant role in a diverse array of abnormalities including growth disorders, cancer, cardiovascular diseases, the metabolic syndrome and type 2 diabetes (15, 17, 23). An increase in the IGF2 concentrations is also associated with higher birth weight (23). Exposure to adverse environmental factors early in life may affect epigenetic patterns of imprinted genes and can lead to enhanced susceptibility to adult diseases (24). This indicates the high importance to understand the role of IGFs during pregnancy and identify the mechanisms underlying the developmental programming of life expectancy (16).
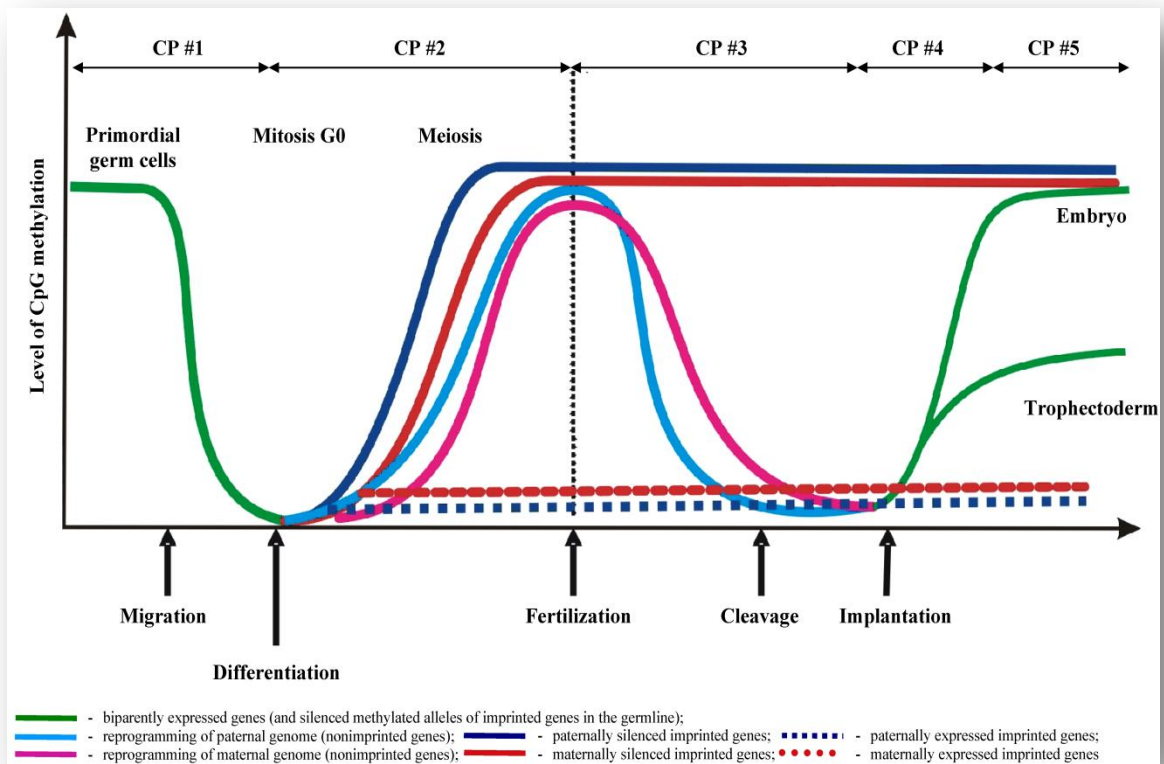
Figure 2 The different phases op de- en remethylation during the epigenetic reprogramming. Several developmental stages show fluctuations of methylation levels in which the implementation of the imprinted marks are coordinated. After the first demethylation, silenced imprinted genes show a continuous high level of methylation while the expressed genes depict stable low methylation levels. Nonimprinted genes display a second de- and remethylation phase. (CP = critical period of reprogramming)

1.5 Hypothesis

In this research, it is hypothesized that prenatal $PM_{2.5}$, PM_{10} and BC pollution exposures are associated with the protein expression and methylation patterns of IGF2 at birth. The first objective is to determine the associations between prenatal $PM_{2.5}$, PM_{10} and BC pollution exposures and IGF2 protein expression at birth. Secondly, we aim to optimise the detection of methylation patterns in the IGF2 gene.

2. Materials and methods

2.1 Study population

The on-going **ENVIR*ON*AGE** (ENVIRonmental influence ON early AGEing) birth cohort includes children born at the East-Limburg Hospital in Genk (Belgium) and their mothers (25). The dispersion of this hospital consists of the province of Limburg in Flanders, Belgium with urban and suburban to rural areas populated by 82 to 743 inhabitants/km² (26).

After birth, cord blood samples were taken and perinatal parameters were gathered, including sex, birth weight and length, birth date and Apgar score. The mothers were asked to complete a **questionnaire** to collect detailed information on place of residence, maternal age, maternal education (low - no high school diploma, middle - high school diploma, high - college or university diploma), pre-pregnancy BMI, smoking status (0 - never smoked, 1 - stopped smoking before pregnancy, 2 - smoked during pregnancy), alcohol consumption during pregnancy (0 - no, 1 – occasionally, 2 - max one glass a day), parity (1 - primiparous, 2 - secundiparous, 3 - multiparous) and ethnicity of the newborn based on the country of origin of the grandparents (0 - 2 out of 4 grandparents are European, 1 - 3 or more grandparents are non-European). Approval of the ethical committees of Hasselt University and the East-Limburg Hospital was obtained, including written informed consent from all participants. The study protocol complied with the Declaration of Helsinki.

In the present study, bio-banked **cord plasma** samples of 516 mother-newborn pairs recruited between February 2010 and June 2015 were randomly selected from a total of 978 pairs in the ENVIR*ON*AGE birth cohort. The dataset was defined by the availability of data and the exclusion of haemolytic cord blood samples.

2.2 Cord blood plasma sample collection

Immediately after delivery, umbilical cord blood was gathered using a syringe and collected in Vacutainer© plastic K2 EDTA Tubes (BD, Franklin Lakes, NJ, USA). After centrifugation for 15 min at 3,200 rpm, the samples were separated into 3 layers (plasma – white blood cells – red blood cells). The plasma was aliquoted into tubes and stored at 80°C until further analysis.

2.3 Determination of prenatal exposure to air pollution

The residential exposure to black carbon, PM_{10} and $PM_{2.5}$ air pollution was determined by using a **spatiotemporal interpolation method** (Kriging) developed by Janssen *et al,* 2008 (27). This process was based on the mothers' home address collected from the questionnaire and confirmed by hospital records. Possible relocation of the mother during pregnancy was taken into account by calculating the trimester-specific exposures from the daily exposure levels at the different residential addresses. Satellite images (CORINE land-cover data set) were used to obtain land-cover data, and pollution data were collected from a governmental stationary monitoring network. These data were coupled to a **dispersion model** that uses emissions from point sources and line sources to accomplish the highest possible accuracy.

The exposure data were obtained as **daily mean** concentrations of $PM_{2.5}$, PM_{10} and BC. Due to a delayed start of the BC measurements, 435 mother-newborn pairs had BC data available for analysis in contrast to 516 pairs for $PM_{2.5}$ and PM_{10} exposure. The possibly critical exposure periods were identified as the 3 trimesters of pregnancy (1st – week 1 to 13, 2nd – week 14 to 26, 3rd – week 27 to delivery), the exposure during the year before delivery and the exposure during the whole pregnancy. The date of conception was predicted according to the first day of the mother's last menstrual period, in combination with the first ultrasound exam. The average daily interpolated concentrations for these periods were used in the statistical analysis.

Leave-one-out cross-validation was applied to assess the overall model performance. $PM_{2.5}$ and PM_{10} exposure measurements noted a spatial temporal variance of 0.8 with 34 monitoring points and 0.7 with 58 monitoring points respectively, while BC listed 14 detection stations and a variance of 0.74(25).

2.4 Quantitative determination of IGF-2 protein using a sandwich enzyme-linked immunosorbent assay (ELISA)

The selected plasma samples were thawed and diluted 1/1000 in sample diluent, as instructed by the manufacturer of the kit, to assure IGF-2 concentrations within the detection range.

The Human IGF2 ELISA kit (protocol 1 in supplemental material) by **Cusabio** (Wuhan, Hubei, China) was used for the measurement of IGF2 proteins in the plasma samples. 100 µl standard solution, blank or sample was added to the wells of a microplate precoated with antibody specific for IGF2. After 2 hours incubation at 37°C, the fluid was removed and 100 µl of Biotin-antibody solution was inserted into each well. The microplate was incubated at 37°C for 1 hour before washing the wells 3 times using 200 µl of Wash Buffer (1x). 100 µl of HRP-avidin solution was pipetted into the wells, followed by 1 hour incubation at 37°C. The wells were washed 5 times. After adding 90 µl TMB substrate, the microplate had to be protected from light and incubated at 37°C for 20 minutes. 50 µl of Stop Solution was added, with an immediate measurement of the optical density at 450 nm and 540 nm using a microplate reader (Fluostar Omega microplate reader, BMG Labtech, Offenburg, Germany).

The standard dilutions and the blank were measured in triplicates. To account for **intervariability** between the plates, 2 different samples were included on each plate and used for normalisation. The **intravariability** was tested using duplicates of 3 random samples on every plate. The placement of the samples was **randomised** to exclude possible aberrant measurements due to varying storage periods.

2.5 Optimization of the detection of methylation patterns in IGF-related genes

Genomic DNA available from placental tissue test samples was bisulphite treated using the EZ DNA methylation Gold^{TM} kit (Zymo research Corp., Irvine, CA) in a final volume of 30 µl M-Elution Buffer. **PCR primers** were determined by literature study combined with the hg19 (GRCh37) UCSC Genome Browser (http://genome.ucsc.edu/) to identify the CpG island promotor regions of interest based on the DNase hypersensitivity regions, transcription binding sites and the percentage of CGs to ascertain a high regulatory activity that may be linked to gene expression. The designed primers defined an optimal distance of 100 to 150 base pairs to produce PCR products with a length of 200 to 300 base pairs.

The amplification of the PCR products was executed using the Pyromark PCR kit (Qiagen Inc., Valencia, CA). A **PCR gradient** was run to determine the optimal amplification temperature. The cycling conditions consisted of an initial activation at 95°C for 15 min, followed by 45 cycles of 30 s at 94°C, 30s at the temperature gradient and 72°C for 30 s, to end with 72°C for 10 min. The amplicons and the 100 bp DNA ladder (GeneRuler, Thermo Fisher Scientific, Waltham, USA) were loaded onto a 2% agarose (UltraPureTM, Invitrogen, Carlsbad, CA) gel stained with GelRed in 1xTAE buffer and separated at 90V for 2h. Visualisation was done by the ImageQuant RT ECL (New life scientific Inc., Cridersville, Ohio).

2.6 Statistical analysis

The association between $PM_{2.5}$, PM_{10} and BC exposure and IGF2 protein expression in cord blood was analysed using **multivariable linear regression models**. SAS 9.4 software (SAS institute Inc., Cary, NC, USA) was used for the statistical analysis. Continuous data were presented as mean ± standard deviation (SD) and categorical data as frequencies and percentages. Statistical significance was defined by a **p-value < 0.05.**

All models were adjusted for gestational age in days (p = 0.04) and highest achieved diploma of the mother (p = 0.01) (low - no high school diploma, middle - high school diploma, high - college or university diploma) based on the significant influence of these factors on the association between the IGF2 and exposure levels. *A priori* chosen covariates were inserted into each exposure model including age of the mother, sex of the newborn (1 – boy, 2 – girl), smoking status (0 - never smoked, 1 - stopped smoking before pregnancy, 2 - smoked during pregnancy), ethnicity of the newborn based on the country of origin of the grandparents (0 - 2 out of 4 grandparents are European, 1 - 3 or more grandparents are non-European) and season in which the delivery occurred (1 – winter, 2 – spring, 3 – summer, 4 – autumn). The year exposure windows were not adjusted for season of delivery as all seasons were equally distributed. Additionally, to determine the independent effect of every trimester of exposure, the trimester-specific models were adjusted for the exposure during the other trimesters. The results are presented as a difference in IGF2 protein expression (ng/mL) for an interquartile range (IQR) increment in exposure of $PM_{2.5}$, PM_{10} or BC.

2.7 Sensitivity analysis

In a sensitivity analysis the associations between the IGF2 protein expression and air pollution exposure were tested while excluding mothers that smoked during pregnancy or experienced hypertension, gestational diabetes or thyroid problems. This also included children born via caesarean section. The **Cook's distances** were determined to exclude possible outliers and residuals.

3. Results

3.1 Characteristics of the study population

Table 1 describes the general characteristics of the **516** mother-newborn pairs involved in this study. The average (± SD) maternal age was 29.1 ± 4.4 years. Mean pre-pregnancy BMI was 24.3 ± 4.8 kg/m² with an averaged net weight gain of 14 ± 5.6 kg during pregnancy. 62.7% of the mothers (n = 326) never smoked cigarettes, 23.1% of women stopped before pregnancy (n =120) whereas 14.2% (n = 74) continued smoking during pregnancy. The majority of mothers (52.7%, n = 272) obtained a higher education degree. Of the participating women, 82.3% did not consume alcohol throughout the pregnancy. In 9.6% of pregnancies complications occurred, while 19 mothers (3.7%) had a C-section. The newborns averaged a birth weight of 3403.9 ± 449.5 g after a mean gestational period of 277.3 ± 9.5 days. In total, the newborn population consisted of 263 boys (50.6%) and 55.2% (n = 287) was primiparous while 34% (n = 177) was secundiparous. 460 newborns (88.5%) were Europeans of Causasian ethnicity. 5 minutes after delivery, the Apgar score was determined 9 or 10 for 90.8% of the newborns (10: 59.6%, 9: 31.2%).

Table 1 Characteristics of mother-newborn pairs (n = 516).

Characteristic	Mean ± SD or n (%)
Maternal	
Age (years)	29.1 ± 4.4
Net weight gain (kg)	14 ± 5.6
Education*	
Low	65 (12.6)
Middle	179 (34.7)
High	272 (52.7)
Smoking status	
Never-smoker	326 (62.7)
Stopped before pregnancy	120 (23.1)
Smoked during pregnancy	74 (14.2)
Alcohol consumption during pregnancy	
No	417 (82.3)
Pregestational BMI (kg/m²)	24.3 ± 4.8
Parity	
1	287 (55.2)
2	177 (34)
≥ 3	56 (10.8)
Cesarean section	19 (3.7)
Pregnancy complications	
Gestational diabetes	20 (3.9)
Hypertension	18 (3.5)
Hypo-/hyperthyroidism	11 (2.2)
Newborn	
Sex	
Boys	263 (50.6)
Ethnicity	
European-Caucasian	460 (88.5)
Gestational age (days)	277.3 ± 9.5
Apgar score 5 min after birth	
7	9 (1.7)
8	39 (7.5)
9	162 (31.2)
10	310 (59.6)
Season of delivery	
Winter	137 (26.3)
Spring	128 (24.6)
Summer	111 (21.4)
Autumn	144 (27.7)
Birth weight (g)	3403.9 ± 449.5

3.2 PM$_{2.5}$, PM$_{10}$ and BC levels

Table 2 shows the mean, 25th and 75th percentile of the residential BC, PM$_{2.5}$ and PM$_{10}$ exposure levels for the different time windows of pregnancy (preconception, trimesters, whole pregnancy, and year). The average exposures during the year before delivery were 0.90 ± 0.27 µg/m³ for BC, 13.59 ± 1.85 µg/m³ for PM$_{2.5}$ and 19.18 ± 2.48 µg/m³ for PM$_{10}$.

Table 2 Ambient air pollution (µg/m³) exposure characteristics for the 516 mothers from the ENVIR*ON*AGE birth cohort included in this study.

Pollutant	Exposure window	Mean ± SD	25th percentile	75th percentile
	Preconception	0.94 ± 0.37	0.65	1.18
	Trimester 1	0.90 ± 0.37	0.61	1.13
	Trimester 2	0.91 ± 0.41	0.61	1.13
Black carbon	Trimester 3	0.94 ± 0.40	0.65	1.14
	Year before delivery	0.90 ± 0.27	0.72	1.07
	Whole pregnancy	0.91 ± 0.29	0.71	1.09
	Preconception	13.75 ± 4.74	9.80889	16.97
	Trimester 1	13.55 ± 4.98	9.54	16.64
	Trimester 2	13.41 ± 4.65	9.66	16.50
PM$_{2.5}$	Trimester 3	13.79 ± 5.39	9.30	17.78
	Year before delivery	13.59 ± 1.85	12.47	14.82
	Whole pregnancy	13.57 ± 2.40	11.61	15.27
	Preconception	19.18 ± 5.02	15.46000	22.57
	Trimester 1	19.19 ± 5.27	15.32	22.28
	Trimester 2	18.96 ± 4.76	15.46	21.76
PM$_{10}$	Trimester 3	19.51 ± 5.59	15.35	22.95
	Year before delivery	19.18 ± 2.48	17.42	20.93
	Whole pregnancy	19.20 ± 2.82	17.05	21.21

3.3 IGF-2 protein expression associated with air pollution exposures

3.3.1 Covariates analysis

IGF2 concentrations in cord blood did not significantly vary by maternal age at delivery (p = 0.11), pre-pregnancy BMI (p = 0.23), alcohol consumption during pregnancy [regular consumer compared to occasional consumer (p= 0.20) or non-consumer (p = 0.14)], smoking status of the mother [never smoked *versus* smoked during pregnancy (p = 0.90), stopped smoking before pregnancy *versus* smoked during pregnancy (p = 0.91)], parity [multiparous compared to secundiparous (p = 0.45) or primiparous (p = 0.46)], date of delivery (p = 0.22), birth weight (p = 0.76), sex (p = 0.24) or ethnicity (p = 0.78) of the newborn, APGAR score 5 min after delivery (p = 0.65) or season at delivery (p = 0.96 for winter, p = 0.93 for spring, p = 0.27 for summer in comparison to autumn). Higher IGF2 concentrations were observed in pregnancies with a longer gestational duration (p = 0.04) and in infants born to mothers with a higher secondary educational degree compared to mothers with a college or university diploma (p = 0.03).

3.3.2 The association between IGF2 protein levels and $PM_{2.5}$, PM_{10} and BC exposure

The average (± SD) cord blood IGF2 concentration in the study population was 438.56 ± 230.31 ng/ml. Before and after adjustment, cord blood IGF2 levels were inversely associated with annual BC, $PM_{2.5}$ and PM_{10} exposure (figure 3). These results were corrected for the gestational duration, highest achieved diploma and age of the mother, sex and ethnicity of the newborn, and smoking status.

For an IQR increment of 0.35 µg/m³ in **annual** maternal BC exposure, the IGF2 expression in cord blood decreased with 55 ng/mL (n = 435, p < 0.0001, 95% CI: -81.91 to -28.15). Corresponding results were found for annual $PM_{2.5}$ exposure (IQR: 2.35 µg/m³) and annual PM_{10} exposure (IQR: 3.51 µg/m³), i.e., -27.8 ng/mL (n = 513, p = 0.04, 95% CI: -53.64 to -1.96) and -35.8 ng/mL (n = 513, p = 0.01, 95% CI: -64.22 to -7.42) in IGF2 expression, respectively. For BC, the maternal exposure during the **whole** pregnancy showed a similar result: an IQR increment of 0.38 µg/m³ resulted in a IGF2 expression decrease of 47 ng/ml (n = 435, p = 0.01, 95% CI: -75.69 to -18.27). The associations between IGF2 protein levels and whole pregnancy exposure for $PM_{2.5}$ (p = 0.11) and PM_{10} (p = 0.10) did not show significant results, despite the presence of a trend. The **Pearson correlation coefficient** (PCC) was checked to explain this lack of a significant association between the IGF2 levels and the exposure during the whole pregnancy while the year before delivery did show a significant link. The PCC of 0.76 for PM2.5, 0.84 for PM10 and 0.93 for BC did not provide justification.

More specific and shorter time periods were correlated to determine the critical stages of exposure effects. The **preconception** exposure windows showed a significant decrease in IGF2 expression of 60.20 ng/mL for BC (n = 370 , IQR: 0.53 µg/m³, p = 0.01, 95% CI: -93.97 to -26.43) and 50.59 ng/mL for PM_{10} (n = 477, IQR: 7.11 µg/m³, p = 0.01, 95% CI: -88.23 to -12.95). For $PM_{2.5}$, the preconception exposure was not significantly associated with IGF2 protein levels (p = 0.15). The **trimester**-specific exposure windows of BC, $PM_{2.5}$ and PM_{10} showed negative but non-significant estimates (p-values > 0.11).

3.4 Sensitivity analysis

After exclusion of mothers that smoked during pregnancy (n = 64 for BC, n = 77 for PM), experienced hypertension (n = 13 for BC, n = 21 for PM), gestational diabetes (n = 16 for BC, n = 23 for PM), thyroid problems (n = 11 for BC, n = 14 for PM) or underwent a caesarean section (n = 14 for BC, n = 22 for PM), the statistical significance of the association between annual BC, $PM_{2.5}$ and PM_{10} exposures and IGF2 levels did not change. For the $PM_{2.5}$ levels in mothers who smoked during pregnancy (p = 0.07) or had to undergo a caesarean section (p = 0.05), the same trend showed **borderline** significance. Including the highest achieved diploma of the father (n = 233) as a covariate did not disrupt the significance of the results. The results of the association between air pollution exposure and IGF2 expression were not affected by the exclusion of potential outliers according to the Cook's Distance test.
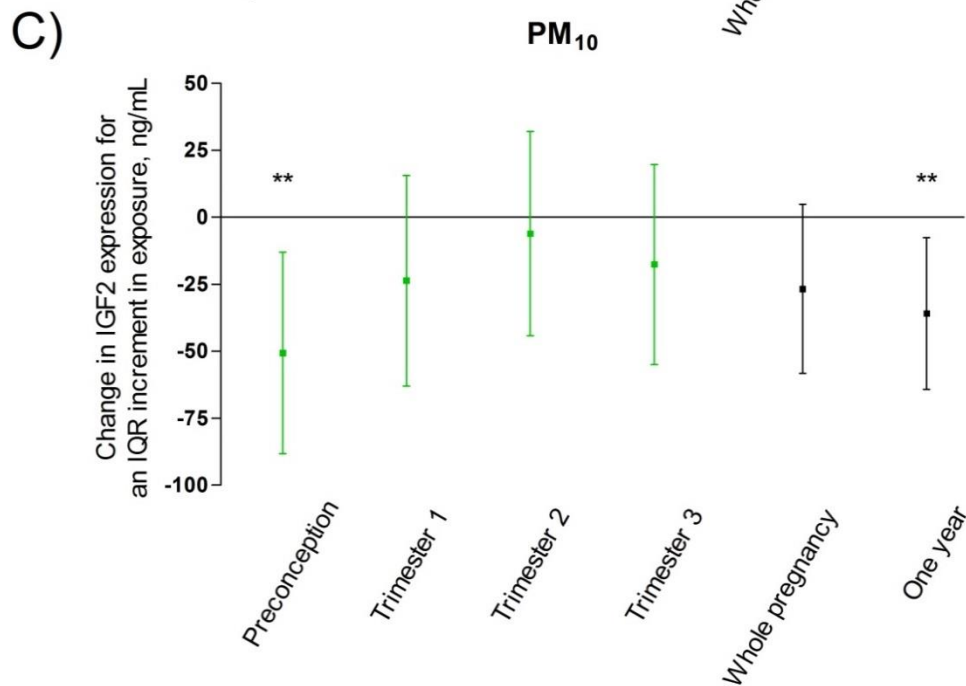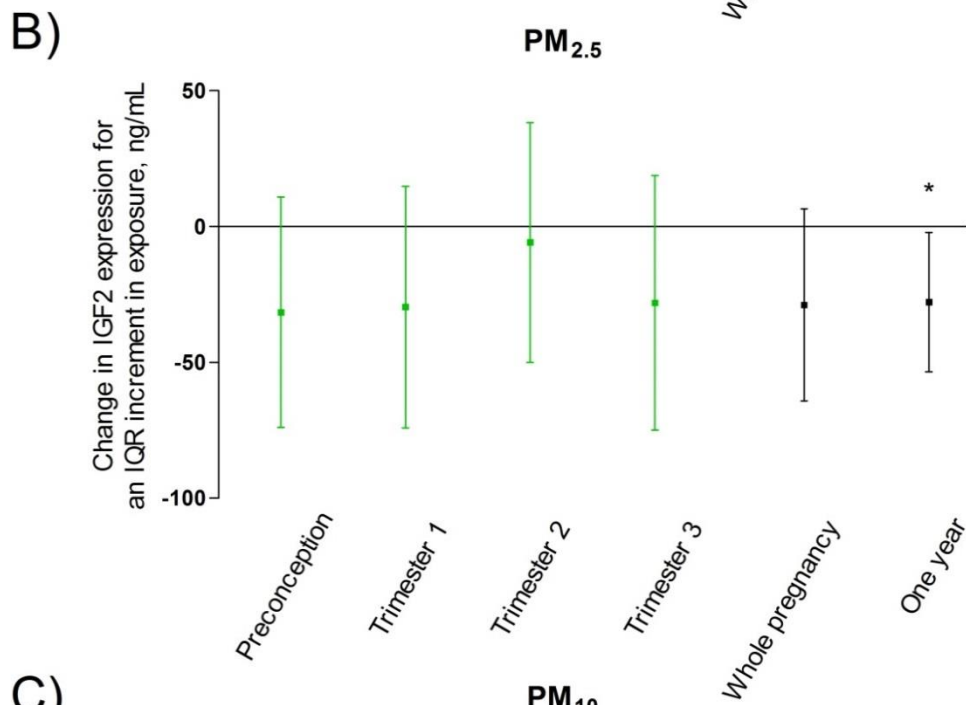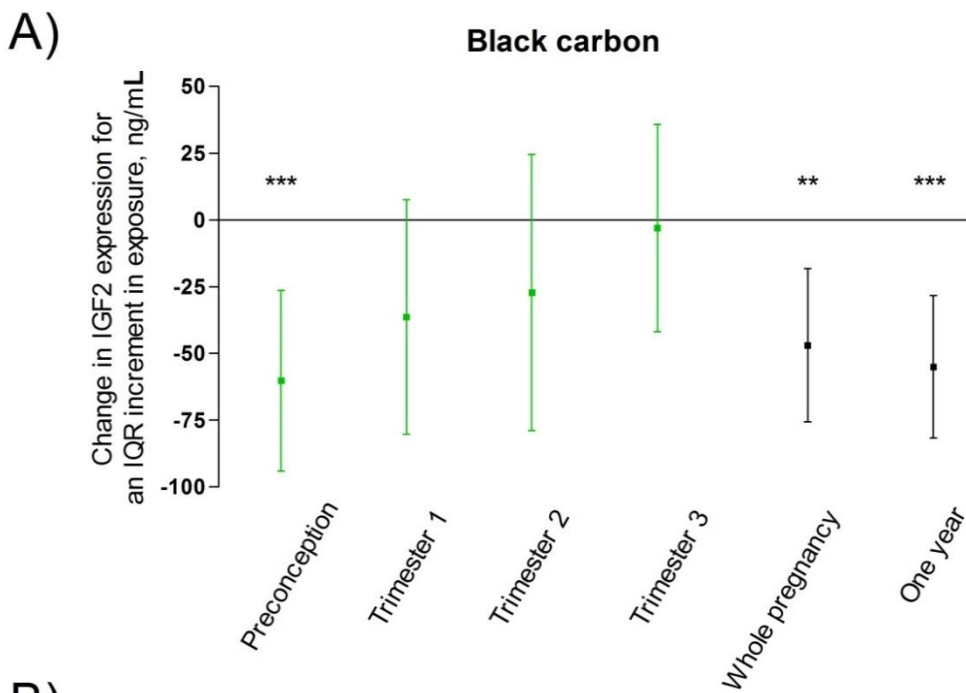
Figure 3 IGF2 protein expression in cord blood associated with BC (A), PM$_{2.5}$ (B) and PM$_{10}$ (C) exposure for different time windows of pregnancy. Estimates (•) are depicted as a difference in IGF2 expression for an IQR increment (μg/m^3) in exposure. The bars depict the corresponding 95% confidence intervals. The IQR for BC, PM$_{2.5}$ and PM$_{10}$ exposure windows are respectively: preconception (0.53, 7.16, 7.11), trimester 1 (0.52, 7.10, 6.96), trimester 2 (0.53, 6.84, 6.31), trimester 3 (0.49, 8.48, 7.60), whole pregnancy (0.38, 3.66, 4.15), and year before delivery (0.35, 2.35, 3.51). Models were adjusted for gestational age in days, season of delivery, sex and ethnicity of the newborn, and age, highest achieved diploma and smoking status of the mother. The significance of the associations is indicated by * (p-value ≤ 0.05), ** (p-value ≤ 0.01) or *** (p-value ≤ 0.001). The correlations of trimester and preconception exposures are depicted in green, those for annual and exposure during the whole pregnancy in black.

3.5 Optimisation DNA methylation analysis of IGF-related genes

Primers designed for the CpG sites within the promotor regions of the IGF1 and IGF2 gene are depicted in table 3. PCR primers were selected after **optimisation** of different PCR primer sets. The intensity of the visualised gel electrophoresis results shown in figure 4 indicates the optimal (+++) amplification temperature for each primer.

Table 3 Properties of the IGF1 and IGF2 primers used for the PCR reaction. Depicted are the forward and reverse sequences, the size of the gene fragment produced and the literature source on which the selection was based.

Gene	Primers (5' – 3')		Source	Product size
	FW	**REV**		
IGF1	TTTGGGGTTTTGTGTTATAAAATTT	TTTAAAAACAACAACTAAATACTACACTAA	(28)	286
IGF2	ATGAATGAGTATTTTTAGGGAAATTGTT	TCCATATCCCCCCTAAATTTAACTTCT	(29)	131
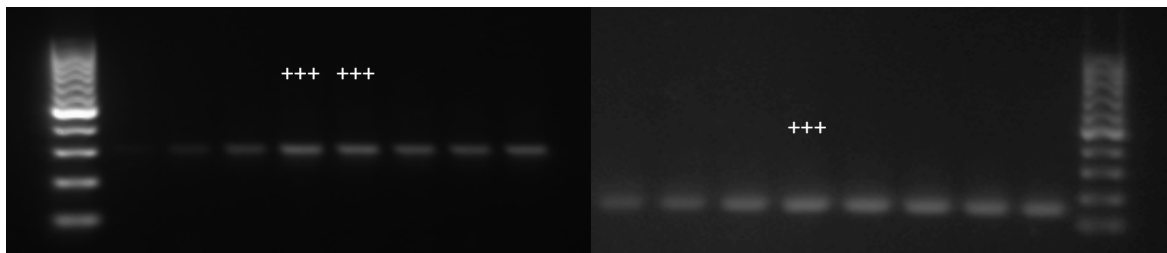
Figure 4 Scans of gel electrophoresis results of DNA-fragments amplified using the IGF1 (left) and IGF2 (right) primers. The product has a size of 286 bp for IGF1 and 131 bp for IGF2. The 100 bp DNA ladder is inserted into the outer lanes. The intensity indicates the efficiency of the amplification and can be used to determine the ideal annealing temperature. +++ indicates the optimal amplification temperature.

4. Discussion

In this study, we evaluated the association between IGF2 protein expression in cord blood and prenatal $PM_{2.5}$, PM_{10} and BC pollution exposure of mother-newborn pairs from the ENVIR*ON*AGE birth cohort. Specific exposure time windows were taken into account, including the preconception, the 3 trimesters of pregnancy, the whole period of pregnancy and the year before delivery. The key finding of this study indicated a **lowered** IGF2 level in association with an increased exposure to annual air pollution particles. Especially the exposure to $PM_{2.5}$, PM_{10} and BC during the preconception window was significantly correlated to the IGF2 proteins in cord blood.

IGF2 is a hormone known for its mitogenic activity, especially during placental and fetal development (15). By silencing the maternal allele of the imprinted gene, DNA methylation regulates the IGF2 expression (23, 30). Methylation patterns are established in the periconceptional period and tend to persist throughout adulthood, making adverse exposure effects in this period crucial towards later in life (23, 31). The **Dutch Hunger Winter study** (DHWS) was the first to observe that intrauterine exposures may have long-lasting consequences for adult health (32). This observation did not only confirm the Barker hypothesis stating that environmental exposures early in life can permanently influence the susceptibility to adult diseases by altering the physiology, metabolism and organ structure of the developing fetus (9), it also initiated an awareness for the importance of exposures during early life, particularly during certain critical developmental windows (32).

Within this concept, it has been shown that intrauterine undernutrition programs for survival in a nutrient-poor environment after birth, leading to postnatal catch-up growth and consequent development of **obesity** when exposed to an energy-rich diet (33). The IGF2 gene may be associated with the altered body composition linked to overweight. Mouse models showed a correlation between lowered IGF2 expression and higher fat mass while experiments in sheep demonstrated that the birth weight of lams is not affected by maternal under- or over-nutrition in the periconceptional period, despite the presence of increased obesity and adverse metabolic parameters later in life (34). Lower circulating IGF2 levels in human adults are associated with increased risk of weight gain and obesity linked to higher subcutaneous adiposity measures (7). In our research, a significant negative association was observed between IGF2 protein levels in cord blood and the prenatal exposure of annual $PM_{2.5}$, PM_{10} and BC. Together with the findings of other studies, our results lead to the hypothesis that air pollution-induced IGF2 expression changes might pose a higher risk to become overweight later in life. However, future studies need to confirm this hypothesis.

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To interpret the importance of prenatal annual exposure to particulate air pollution, we correlated IGF2 levels to the exposures during **critical exposure windows** within the year. These results indicated the preconception period and the 1st trimester as the most important exposure windows. The relevance of the preconception period can be explained by IGF2 being an imprinted gene that is paternally expressed. The exposure to air pollution elements before conception might be strongly linked to the IGF2 levels that influence placental and fetal growth and development. Since the IGF2 gene is paternally imprinted, this finding suggests that air pollution exposure might influences the unborn child's susceptibility to adult diseases before its fertilization, by affecting the IGF2 gene status of the **father**. Further research connecting these findings to paternal IGF2 gene data and the health status in adulthood is required to confirm possible links.

Particularly **BC** exposure showed strong correlations to IGF2 expression in cord blood. BC particles may strongly influence IGF2 levels because its diameter of less than 0.01 μm makes it able to penetrate the body more deeply than larger particulates and even cross the blood-brain-barrier leading to higher damage to internal systems (2, 8, 35). Secondly, BC is a **traffic-related** anthropogenic air pollutant of which diesel exhaust is the most dominant source. The concentration of BC is a strong indicator of the influence of traffic density on the air quality (36). These results might indicate that higher traffic pollution will influence human health more than increased occurrence of processes that produce larger pollutants.

It is not yet possible to investigate whether the decrease of IGF2 levels indicates a higher risk for certain health outcomes later in life. We can only shape **hypothetical** connections based on literature concerning the known functions of IGF2. One of its functions is controlling somatic tissue growth through balancing cell proliferation and apoptosis. Although this balance tends to lean towards a growth promoting effect, several diseases display a complicated etiology induced by over- or underexpression of IGF2 (34, 37). **Carcinogenesis** for example is linked to the overexpression of IGF2 caused by loss of imprinting (LOI) that leads to biallelic expression (15). IGF2 levels also play a role in developmental abnormalities of the cerebellum and the hippocampus that are linked to **attention deficit hyperactivity disorder** (ADHD). In early-onset persistent ADHD, the levels of IGF2 at birth predict the severity of ADHD symptoms in childhood. The same decreased IGF2 levels associated with air pollution exposure are seen in case of a prenatal high-fat and high-sugar diet, indicating that diet may be correlated to IGF2 expression (38). Research on human and rat prostate tissues also showed an increase in IGF2 levels with **aging**. This leads to the hypothesis that cellular aging causes altered gene expression due to changes in the imprinting status of IGF2 and is supported by the existence of IGF2 LOI in aging-associated cancers like colon cancer and prostate cancer (17).

Similarly, increased IGF2 levels are seen in cases of **macrosomia** in comparison to babies with a normal birth weight (39). Despite this observation, the association between IGF2 levels and birth weight is not clear due to contradictory results. Some studies show no association while others identify a negative correlation between IGF2 and birth weight or report a higher birth weight linked to elevated IGF2 levels in cord blood (40). Igf2-deficient mice display severe growth restriction at delivery, weighing 40% less than healthy litter mates (31, 34, 41). This finding was supported by a study in newborns in which elevated IGF2 cord blood concentrations were associated with higher birth weight (23). The combination of both studies indicates that decreased IGF2 levels may be associated with lower birth weight. In our study, the corrected association between **birth weight** and IGF2 levels in cord blood showed no significance (p = 0.18) which might be explained by the possibility that intrauterine exposures that changed IGF2 levels in well-nourished populations are subtle and do not cause great birth weight changes (34). In addition, the ENVIR*ON*AGE birth cohort also did not find an association between particulate air pollution and birth weight (p = 0.41 for year BC, p = 0.82 for $PM_{2.5}$ and p = 0.56 for PM_{10}). However, an unchanged birth weight does not necessarily mean that the intrauterine exposures will have no long-term consequences. Children born to mothers only exposed to undernutrition during early gestation had normal birth weights, despite experiencing increased rates of obesity later in life (32).

The main **limitation** of this study is related to the determination of the $PM_{2.5}$, PM_{10} and BC **exposure levels**. Since the measurements are based on home addresses, the model assumes the mother does not leave the house during pregnancy. Without taking social activities into account, this is still unrealistic as most mothers are employed at different and sometimes distant locations. Due to the varying personal behaviour, it is impossible to include these variations into the exposure determinations. Simultaneously, the exclusion of this diversity prevents confounding bias caused by personal differences. Nevertheless, possible moving of the mother during pregnancy is taken into account. The levels of air pollution also vary over the course of the day, for example higher levels of pollutants are detected during heavy traffic (8).

The use of daily mean concentrations does not correctly depict exposure differences between mothers that avoid rush hours and those who are exposed to those peak pollutant levels. The positioning of the measuring stations also raises questions in situations where they are located in a particularly green area, which obviously does not represent the air pollution levels near busy roads or heavy industry. This might indicate that the actual levels are higher, leading to a strengthening of the association we detected. Despite the less optimal context of the exposure measurements, the highest possible accuracy is accomplished by coupling the data to a dispersion model. The exposure models did explain more than 0.70 of the spatial and temporal variability.

Another limitation is the absence of a **causal link** between the IGF2 levels and air pollution exposures due to the epidemiologic nature of this study. Lastly, the possibility to correlate these results to **health outcomes** would strongly contribute to the study. Unfortunately, these data are not yet available. The **strength** of this study consists of the **size** of the study population (n = 516) and the extensive information readily available in the ENVIR*ON*AGE birth cohort. Secondly, by **randomisation** of the samples on the plates and using internal controls for intra- and inter plate variation during the ELISA measurements, we excluded possible aberrant measures due to varying storage periods or technical variation.

5. Conclusion and future perspectives

The IGF2 levels in cord blood are negatively associated with the annual and preconception exposure to $PM_{2.5}$, PM_{10} and BC. The relevance of the preconception period together with the imprinting nature leading to IGF2 to only be paternally expressed, might indicate a more prominent influence by the **father's exposure** on IGF2 levels of the unborn child. The strongest correlation to IGF2 is attributed to BC that consists of particles with a diameter of less than 0.01 µm, showing the relevance of traffic density in studies investigating the effects of air pollution on human health. Further research is required to elucidate the health consequences later in life associated with these changes in air pollution-induced IGF2 expression.

A possible follow-up study might be aimed at completing the **optimisation** of the DNA methylation analysis to investigate the correlation between the IGF2 methylation status, the IGF2 levels and air pollution exposure. Further elucidating the influence of **paternal factors** on the IGF2 levels and the association between the IGF2 gene status of the father and IGF2 levels in cord blood may be interesting as well. Unfortunately, studies including the **adult health data** of the involved children are preserved for the future since the current study population consists of toddlers and their mothers.

Supplemental material

Protocol 1

Human Insulin-Like Growth Factors 2 (IGF-2) ELISA kit

Name: Saskia Put
Original protocol:
Cusabio IGF-2 ELISA Kit (Catalog Number CSB-E04583h)
Last date modified:
- 22/02/2017
Special safety and hazardous waste disposal remarks + lab zone: • Wear a lab coat, mask, cap and laboratory gloves at all times during this procedure.
Lab zone: Molecular Epidemiology DNA lab (G27d)
Safety   **Relevant MSDS sheets:**   • Biotin-antibody (diluent)   • HRP-avidin (diluent)   • Sample Diluent   • TMB Substrate   • Stop solution   **Extract from MSDS sheets:**   Biotin-antibody: After inhalation: supply fresh air; consult a doctor in case of complaints   After ingestion: if symptoms persist consult doctor   After skin exposure: product does not irritate skin   After eye exposure: rinse opened eye for several minutes under running water   HRP-avidin: After inhalation: remove to fresh air.   After ingestion: wash mouth with plenty of water. Get medical attention if stomach discomfort persists.   After skin exposure: rinse thoroughly with water. Wash with soap.   After eye exposure: immediately flush eyes with water for at least 15 minutes. Seek medical attention if irritation persists.   Sample Diluent: After inhalation: remove to fresh air.   After ingestion: wash mouth with plenty of water. Get medical attention if stomach discomfort persists.   After skin exposure: rinse thoroughly with water and soap.   After eye exposure: immediately flush eyes with water for at least 15 minutes. Seek medical attention if irritation persists.   TMB Substrate: Hazard identification: mild oxidizing agent.   After inhalation: remove to fresh air. If breathing is difficult, get medical attention.

After ingestion: if person is conscious, wash out mouth with water. Get medical attention.

After skin exposure: wash skin with soap and water. If irritation develops or persists, get medical attention.

After eye exposure: immediately flush eyes with water for at least 15 minutes. Get medical attention.

Stop solution:

Hazard identification: acid solution. Corrosive to body tissues and slightly toxic by ingestion.

After inhalation: remove to fresh air or supply with oxygen. If breathing is difficult, get medical attention. If not breathing, give artificial respiration.

After ingestion: if person is conscious, wash out mouth with water. Get medical attention.

After skin exposure: wash skin with soap and water. If irritation develops or persists, get medical attention.

After eye exposure: immediately flush eyes with water for at least 15 minutes, while lifting lids occasionally. Get medical attention.

Follow general safety rules for all solutions:

Harmful if swallowed, irritating to eyes and skin. Keep away from food and drinks.

Materials and reagents
• ELISA kit - Cusabio
• Distilled water

Equipment
• Microplate reader (measurement absorbance at 450 nm)
• Incubator (37°C)
• Absorbent paper
• Centrifuge
• Micropipettes – 1000 µl, 100 µl, 10 µl
• Multichannel automated pipette
• Disposable 1.5 mL tubes
• Isomobox with ice

Remarks
• Do not mix or substitute reagents with those from other lots or sources.
• If samples generate values higher than the highest standard, dilute the samples with Sample Diluent and repeat the assay.
• Never touch the bottom of the assay plate with any materials to prevent damaging the antibody coating.
• Grossly hemolyzed samples are not suitable to use in this assay.
• Fresh samples without long storage time are recommended for this test.
• Do NOT vortex to mix. Gently flicking will prevent aberrant results.

Preparations before starting
• Set incubator to 37°C.
• Check Wash Buffer: if the formation of crystals has occurred, warm it up to room temperature and mix gently until the crystals completely dissolve.

Procedure							

Sample collection and storage

- Collect **plasma** using EDTA, or heparin as an anticoagulant.
- Centrifuge for 15 minutes at 1000 x g at 2-8°C within 30 minutes of collection.
- Form aliquots to avoid repeated freeze-thaw cycles.
- Store samples at -20°C or -80°C.

Sample preparation

- Bring all reagents and samples to room temperature.
  - → Thaw samples on ice: flick to help them thaw if desired.
  - → Kit can be kept at room temperature after thawing.
- Centrifuge samples again after thawing (15 min at 1000g).
  - → Not necessary if aliquots are used.
- Predict estimated concentration of samples: dilute if the values are not within the range of the standard curve.
  - → Plasma samples require a 1000-fold dilution into Sample Diluent. This can be achieved by first adding 5 µl sample into 95 µl Sample Diluent, followed by the addition of 5 µl of the obtained solution to 245 µl Sample Diluent.

Reagent preparation

Note: prepare sufficient amounts of reserve volume to compensate for the loss of fluid due to the formation of bubbles.

- First centrifuge the **Standard vial** at 8000 rpm for 30s. Next add 1.0 ml of Sample Diluent to the Standard to produce a stock solution of 4000 pg/ml. Flick the vial to fully dissolve the powder and let it incubate for 15 minutes with gentle agitation.
  - → The standard has to be prepared fresh for each assay, and used within 4 hours.
- Prepare the **Wash Buffer** (1x) by diluting 20 ml of Wash Buffer Concentrate (25x) into distilled water to a total of 500 ml (execution in 2h incubation step).
  - → Wash Buffer can be produced in bulk due to its longer shelf life.
- Centrifuge the **Biotin-antibody vial** before opening. Prepare a 100-fold diluted solution consisting of 10 µl Biotin-antibody in 990 µl Biotin-antibody Diluent.
- Centrifuge the **HRP-avidin vial** before opening. Prepare a 100-fold dilution of 10 µl HRP-avidin in 990 µl HRP-avidin Diluent.
- Make **serial dilutions of the Standard** by adding 350 µl Sample Diluent and 350 µl of the former diluted solutions. Flick and short spin the tube before every transfer. The undiluted standard is used as the high standard, while Sample Diluent will be the zero.
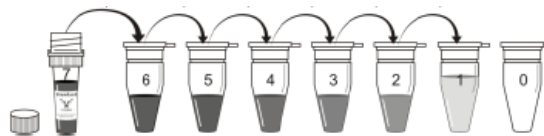
OR

Tube	S7	S6	S5	S4	S3	S2	S1	S0
Dilution in 350µl Sample Diluent	350µl **Standard**	350µl **S7**	350µl **S6**	350µl **S5**	350µl **S4**	350µl **S3**	350µl **S2**	350µl Sample Diluent
pg/ml	4000	2000	1000	500	250	125	62.5	0

Assay procedure

Note: NEVER let the wells dry out completely.

Note: avoid bubbles in the wells by applying reverse pipetting where possible.

- Remove wells that will not be used from the plate, seal them back into the pouch and store at 4°C.
- Mark the columns according to the order in which they're used.
  (safety in case of detachment of the wells from the plate during the process)
- Add **100µl standard or sample** to the wells. Cover with an adhesive strip and **incubate at 37°C for 2 hours**.
- Remove the liquid from the wells by inverting and blotting the plate against clean paper towels.
- Add **100µl Biotin-antibody solution** to each well and cover with an adhesive strip. **Incubate 1 hour at 37°C**.
- Empty the plate onto paper towels. Wash the wells by adding **200 µl Wash Buffer**. After 2 minutes, remove the liquid completely by blotting the plate against paper towels. Repeat this for a total of three washes. *Note*: make sure all fluid is removed before proceeding to the next step.
- Add **100µl HRP-avidin solution** to each well and cover with an adhesive strip. **Incubate 1 hour at 37°C**.
- **Wash the wells** five times as seen earlier.
- Add **90 µl TMB substrate** to every well. Protect the plate from light, then incubate it for **15-30 minutes at 37°C**.
- Add **50 µl Stop Solution** to each well. Gently tap the plate to ensure thorough mixing.
- Within **5 minutes**, the optical density has to be measured at **450 nm using a microplate reader**. Measure again at 540 nm. Correct for optical imperfections by substracting the readings at 540 nm from the readings at 450 nm.

Calculation of the results

- Substract the blank value from all other values.
- Construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration of the y-axis.
- If samples have been diluted, the concentration read from the standard curve needs to be multiplied by the dilution factor.

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