DOCTOR OF SCIENCES

Air pollution exposure in early life
Placental molecular changes and clinical microvascular outcomes

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AIR POLLUTION EXPOSURE IN EARLY LIFE: PLACENTAL MOLECULAR SIGNATURES AND CLINICAL MICROVASCULAR OUTCOMES

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SUMMARY

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According to the 'DOHaD' or 'Barker' hypothesis, environmental factors to which the fetus is exposed during pregnancy can induce prenatal molecular changes, which in turn can contribute to an increased risk of developing chronical disease conditions later in life. Air pollution is such an environmental factor to which various adverse health conditions have been associated. Already very early during fetal development, black carbon particles can be found at the fetal side of the placenta and molecular signatures show how the effects of air pollution can manifest themselves in placental tissue, with potential profound consequences for human health. It is well known that air pollution exposure has a great effect during the earliest phases of life. However, to date, little has been described about the clinical health consequences of prenatal and postnatal exposure to ambient air pollution in young children.

Within this PhD project we discuss several of the links in the process from environmental exposure to early molecular changes and the eventual clinical outcomes in young children. In the general introduction we discuss the background of this work, with specific attention for a summary of the placental molecular changes that have already been associated with *in utero* air pollution exposure and the potential effects on health and disease during early childhood that have been described in this context. Subsequently, we develop the different branches of research investigated within this PhD project in 2 main parts. The first part consists of 3 chapters focused on molecular measurements in placental tissue differentially exposed to air pollution during pregnancy. The first chapter discusses the optimization process for the identification of the placental proteome by means of label-free nano tandem mass spectrometry. In the second chapter we have used this optimised technique to investigate the association between prenatal exposure to black carbon air pollution and the proteomic changes in the placenta. In the final chapter of the first part we focussed on an *a priori* selected target found in placental tissue, the TRPC6 cation channel, and its association with $PM_{2.5}$ air pollution exposure during pregnancy.

The second part of this PhD project consists of 2 chapters that concentrate on the impact of prenatal air pollution exposure on the retinal microcirculation at the age of 4. In the first chapter we have examined the association between retinal vessel characteristics, more specifically the diameter and tortuosity of the vessels,

between 4 and 5 years of age and both prenatal and postnatal exposure to $PM_{2.5}$ and NO_2 air pollution. In the final chapter, we elaborate on the association between retinal microvascular characteristics and the neurological development of the children in our study.

Table 1 gives a summary of the studies discussed within this PhD project, with regard to what is already known on the subject from literature, to what the study adds to the knowledge of the respective research domain and to the future perspectives that result from the conclusions of each study.

Table 1. Summary of this doctoral dissertation

Part 1: Placental molecular effects of prenatal air pollution exposure			
Chapter	**What is known**	**What this study adds**	**Conclusions and perspectives**
Chapter 1	• Studies on placental proteomics use different protein extraction and digestion techniques • Study results can be combined through unified techniques	• Placental protein identification is increased by: o Extra washing with PBS o Mechanical tissue disruption o FASP-based liquid digestion	• This optimized technique can be implied in biomarker research • Future technique improvements should be monitored and tested
Chapter 2	• Black carbon particles can be found in placental tissue as early as 12 weeks of pregnancy • Prenatal exposure to air pollution can cause molecular changes in the placenta	• 13 proteins are at least 2-fold overexpressed in the placentas of women exposed to high black carbon levels during pregnancy • 5 pathways were enriched, involved in extracellular matrix receptor interaction, intermediate filament organization, fibrin clot formation, haemostasis, sodium ion transport regulation	• High black carbon exposure levels during pregnancy alter placental protein expression for multiple pathways • Further research should verify our findings on other -omics levels, and should investigate the clinical impact of these changes

Continued

Table 1. Continued

Part 1: Placental molecular effects of prenatal air pollution exposure			
Chapter	**What is known**	**What this study adds**	**Conclusions and perspectives**
Chapter 3	• TRP channels are sensors of oxidative stress and chemical stressors • TRPC6 channels transport Ca^{2+} in the placenta	• A 5 µg/m³ increment in $PM_{2.5}$ exposure during the third trimester of pregnancy is associated with a 26.6% increase in placental *TRPC6* expression • *TRPC6* mRNA and protein expression are inversely correlated, and TRPC6 protein expression is negatively associated with $PM_{2.5}$ exposure between weeks 3-15 and weeks 27-35 of pregnancy	• TRPC6 channel expression is affected by trimester-specific $PM_{2.5}$ exposure, both on the mRNA and protein level • The link with placental Ca^{2+} transport and other TRP channels should be further investigated

Continued

Table 1. **Continued.**

Part 2: Microvascular changes associated with (prenatal) air pollution exposure			
Chapter	**What is known**	**What this study adds**	**Conclusions and perspectives**
Chapter 4	• The microcirculation undergoes organ-specific perinatal maturation • (Prenatal) air pollution exposure is associated with cardiovascular disease	• Increased prenatal $PM_{2.5}$ and NO_2 exposure widen the arteriolar and venular retinal diameter • Increased NO_2 exposure during the third trimester and entire pregnancy increases retinal vessel tortuosity	• Prenatal $PM_{2.5}$ and NO_2 exposure increase retinal vessel diameter and tortuosity at the age of 4 to 5 years • Future research should elucidate whether these microvascular changes reflect cardiovascular health
Chapter 5	• The retinal microvasculature is a mirror of cerebrovascular conditions • Changes in retinal microvasculature are associated with IQ at the age of 11 years.	• Wider venules and increased vessel tortuosity were related to a worse short-term visual recognition memory performance • Wider retinal venules are associated with slower reaction time	• Retinal vessel characteristics reflect neurological development early in life • These associations should be confirmed at a later age.

SAMENVATTING

SAMENVATTING

Volgens de 'DOHaD' of 'Barker' hypothese kunnen omgevingsfactoren waaraan een foetus wordt blootgesteld tijdens de zwangerschap prenatale moleculaire veranderingen induceren, dewelke kunnen bijdragen tot een verhoogd risico op chronische aandoeningen later in het leven. Luchtvervuiling is zo een omgevingsfactor waaraan reeds verschillende negatieve gezondheidsrisico's gelinkt werden. Al tijdens de vroege ontwikkeling van de foetus zijn roetdeeltjes terug te vinden aan de foetale kant van de placenta en is te zien hoe de effecten van luchtvervuiling zich manifesteren op moleculair gebied, met mogelijke ingrijpende gevolgen op de humane gezondheid. Ondanks de wetenschap dat vooral in de vroegste fasen van het leven de effecten van omgevingsblootstellingen zoals luchtvervuiling een grote impact hebben, is er over de klinische effecten van luchtvervuiling in jonge kinderen tot op heden weinig beschreven.

In dit proefschrift bespreken we enkele schakels in het proces van omgevingsblootstelling, tot moleculaire verandering, en de uiteindelijke klinische uitkomst in jonge kinderen. In de algemene introductie wordt de achtergrond van dit werk besproken, met specifieke aandacht voor een samenvatting van de moleculaire veranderingen in de placenta die reeds geassocieerd werden met blootstelling aan luchtvervuiling tijdens de zwangerschap, en de gezondheidseffecten die hieraan gelinkt worden. Vervolgens wordt een uiteenzetting van ons werk besproken binnen 2 onderdelen. Het eerste onderdeel bestaat uit 3 hoofdstukken, dewelke als gemeenschappelijke noemer moleculaire metingen in de placenta draagt. Hoofstuk 1 bespreekt het optimalisatieproces voor de bepaling van het proteoom in de placenta door middel van label-vrije nano tandem massaspectrometrie. In het tweede hoofdstuk gebruikten we deze geoptimaliseerde techniek om de associatie te onderzoeken tussen blootstelling aan roetdeeltjes tijdens de zwangerschap en de effecten die hiervan te zien zijn op eiwitniveau in de moederkoek. Hoofdstuk 3 zoemt in op een specifiek doelwit dat te vinden is in placentaal weefsel en dat *a priori* geselecteerd werd, het TRPC6 ionenkanaal, en de associatie hiervan met $PM_{2.5}$ luchtvervuiling tijdens de zwangerschap.

Het tweede onderdeel van dit werk bestaat uit 2 hoofdstukken, die dieper ingaan op de effecten van luchtvervuiling op de microcirculatie van de retina in kinderen

op de leeftijd van 4 jaar. In het eerste hoofdstuk bespreken we het effect van $PM_{2.5}$ en NO_2 luchtvervuiling op de diameter en de kronkeligheid van de bloedvaten in de retina. In het tweede hoofdstuk binnen dit onderdeel bespreken we de relatie tussen de kenmerken van de retina-vasculatuur en de neurologische ontwikkeling van de 4-jarige kinderen in onze studie.

Tabel 1 geeft een samenvatting van elk van de besproken studies binnen dit proefschift, met betrekking tot wat reeds geweten is uit de literatuur, hetgeen de studie toevoegt aan het onderzoeksdomein van epidemiologisch omgevingsonderzoek en de toekomstige perspectieven tot dewelke de bevindingen van elke studie bijdragen.

<u>Tabel 1.</u> Samenvatting van deze doctoraatsdissertatie

Deel 1: Moleculaire tekenen in de placenta van prenatale blootstelling aan luchtvervuiling			
Hoofdstuk	**Wat is er gekend**	**Wat voegt de studie toe**	**Conclusies en perspectieven**
Hoofdstuk 1	• Studies over het proteoom van de placenta gebruiken verschillende technieken voor eiwit extractie en verwerking • Studieresultaten kunnen vergeleken worden door middel van uniforme technieken	• Verhoogde eiwitidentificatie door: o Extra wassen met PBS o Mechanische weefsel disruptie o FASP-gebaseerde peptide productie in vloeistof	• Deze geoptimaliseerde techniek kan gebruikt worden in onderzoek naar ziektemerkers • Verbeteringen van technieken moeten opgevolgd en onderzocht worden
Hoofdstuk 2	• Roetdeeltjes kunnen teruggevonden worden in placentaweefsel, reeds vanaf de 12de week van de zwangerschap • Prenatale blootstelling aan luchtvervuiling kan moleculaire veranderingen in de placenta veroorzaken	• 13 eiwitten vertonen een minstens tweevoud overexpressie in de placenta's van vrouwen die blootgesteld werden aan veel roet tijdens de zwangerschap • 5 processen zijn hierbij uitgelicht, betrokken bij extracellulaire matrix receptor interactie, intermediare filament organizatie, hemostase, fibrinestolling en de regeling van natrium transport	• Hoge blootstelling aan roetdeeltjes tijdens de zwangerschap verandert de expressie van eiwitten in de placenta • Toekomstig onderzoek moet de gevonden resultaten verifiëren op andere moleculaire niveaus, en de klinische impact ervan onderzoeken

Vervolgd

Tabel 1. **Vervolg**

Deel 1: Moleculaire tekenen in de placenta van prenatale blootstelling aan luchtvervuiling			
Hoofdstuk	**Wat is er gekend**	**Wat voegt de studie toe**	**Conclusies en perspectieven**
Hoofdstuk 3	• TRP-kanalen zijn sensoren voor oxidatieve stress en chemische omgevingsfactoren • TRPC6 kanalen transporteren Ca^{2+} in de placenta	• Een 5 µg/m³ verhoogde blootstelling aan $PM_{2.5}$ in het derde trimester van de zwangerschap verhoogt de placentale expressie van *TRPC6* met 26.6% • *TRPC6* mRNA en eiwit expressie hebben een omgekeerde correlatie, en TRPC6 eiwit expressie is negatief geassocieerd met $PM_{2.5}$ blootstelling tussen week 3-15 en week 27-35 van de zwangerschap	• TRPC6 expressie wordt beïnvloed door trimester-specifieke $PM_{2.5}$ blootstelling, zowel op mRNA- als op eiwitniveau • De link met Ca^{2+} transport in de placenta en met andere TRP kanalen moet verder onderzocht worden

Vervolgd

Tabel 1. **Vervolg**

Deel 2: Microvasculaire veranderingen in associatie met (prenatale) blootstelling aan luchtvervuiling			
Hoofdstuk	**Wat is er gekend**	**Wat voegt de studie toe**	**Conclusies en perspectieven**
Hoofdstuk 4	• De microcirculatie ondergaat orgaan-specifieke ontwikkeling • (Prenatale) blootstelling aan luchtvervuiling is geassocieerd met cardiovasculaire aandoeningen	• Verhoogde prenatale $PM_{2.5}$ en NO_2 blootstelling verwijden de bloedvatdiameter in de retina • Verhoogde NO_2 in het derde trimester en volledige zwangerschap verhoogt de kronkeling van retinabloedvaten	• Prenatale $PM_{2.5}$ en NO_2 blootstelling beïnvloeden de retinabloedvaten op de leeftijd van 4 tot 5 jaar. • Onderzoek moet uitwijzen of deze veranderingen de (toekomstige) cardiovasculaire gezondheid weerspiegelen
Hoofdstuk 5	• De microvasculatuur van de retina is een weerspiegeling van de vasculaire toestand in de hersenen • Veranderingen in vasculatuur van de retina zijn reeds geassocieerd met IQ op de leeftijd van 11 jaar.	• Verbreedde venulen en verhoogde kronkeling van de retinabloedvaten waren gelinkt met een slechtere score van het korte-termijn visueel herkenningsgeheugen • Verbrede venulen zijn geassocieerd met een tragere reactiesnelheid	• Veranderingen in de retinabloedvaten reflecteren neurologische ontwikkeling vroeg in het leven • Deze associaties moeten opgevolgd en bevestigd worden op latere leeftijd.

LIST OF ABBREVIATIONS

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3-NTp:	3-nitrotyrosine
8-oxodG:	8-oxo-2'-deoxyguanosine
ACN:	Acetonitrile
AHH:	Aryl hydrocarbon hydroxylase
AOC1:	Amiloride-sensitive amine oxidase [copper-containing] 1
ATP2B4:	Plasma membrane calcium-transporting ATPase 4
BC:	Black carbon
BDNF:	Brain-derived neurotrophic factor
BMI:	Body mass index
CFHR1:	Complement factor H-related protein 1
CI:	Confidence interval
CIMT:	Carotid intima-media thickness
COL4A2:	Collagen type 4 alpha-2 chain
CRAE:	Central retinal arteriolar equivalent
CRVE:	Central retinal venular equivalent
CYP1A1:	Cytochrome P450 1A1
D0:	Capacity dimension
D1:	Information dimension
D2:	Correlation dimension
DOHaD :	Developmental Origins of Health and Disease
DTT:	Dithiothreitol
ECOD:	7-ethoxycoumarin O-deethylase
ECM:	Extracellular matrix
ENVIR*ON*AGE:	Environmental Influences on Early Ageing
FGL1:	Fibrinogen-like protein 1
GP1BB:	Platelet glycoprotein Ib beta chain
GST:	Glutathione S-transferase

GSTM1:	Glutathione S-transferase M1
IAA:	Iodoacetamide
IQR:	Interquartile range
KRT17:	Keratin, type I cytoskeletal 17
LC-MS/MS:	Liquid chromatography tandem mass spectrometry
LEP:	Leptin
MAP:	Mean arterial pressure
miRNA:	MicroRNA
MT:	Metallothionein
mtDNA:	mitochondrial DNA
MYO1B:	Unconventional myosin-1b
NAT2:	N-acetyl transferase 2
NO_2:	Nitrogen dioxide
NOTUM:	Palmitoleoyl-protein carboxylesterase NOTUM
OD:	Optic disk
PAH:	Polycylic aromatic hydrocarbons
PBS:	Phosphate buffered saline
PC:	Principal component
PECO:	Population, Exposure, Comparator, and Outcome elements
PKP2:	Plakophilin-2
PM:	Particulate matter
$PM_{2.5}$:	Particulate matter with a diameter smaller than 2.5 µm
PM_{10}:	Particulate matter with a diameter smaller than 10 µm
qPCR:	quantitative polymerase chain reaction
ROS:	Reactive oxygen species
SERPINE2:	Serine protease inhibitor; glia-derived nexin
SD:	Standard deviation

LIST OF ABBREVIATIONS

SNP:	Single nucleotide polymorphism
SO_2:	Sulfur dioxide
TAC3:	Tachykinin-3
TFA:	Trifluoroacetic acid
TI:	Tortuosity index
TIMP3:	Matrix metalloproteinase 3
TRPC6:	Transient receptor potential cation channel 6
UA:	Urea acetate
UFP:	Ultrafine particles
WHO:	World Health Organization

TABLE OF CONTENTS

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GENERAL INTRODUCTION

1. The developmental origins of health and disease

According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, also known as the Barker hypothesis, environmental exposures posed upon a mother during pregnancy can result in permanent molecular changes in her growing fetus. These changes can prepare the offspring for similar (adverse) conditions after birth and potentially increase its survival rate.[1] However, many studies have shown that *in utero* exposures can also result in a higher chance of developing chronic diseases later in life, leading to increased morbidity and mortality. In the early 1990's, Professor David Barker was the first to recognize the link between prenatal exposures and its clinical consequences later in life, describing an association between lower birth weight and an increased chance of developing cardiovascular disease.[2,3] Another example of an implication of the DOHaD hypothesis is the Dutch 'Hunger Winter' of 1944-1945, during which people in The Netherlands were exposed to extreme famine. Children born from pregnancies that were carried out in this period showed various detrimental conditions at a later age: especially in women, prenatal famine resulted in a dyslipidemic pattern characterised by higher cholesterol and triglyceride levels.[4] Furthermore, this population showed a higher mortality rate in late adulthood from natural and external causes when they were exposed to severe malnutrition during the first trimester of pregnancy.[5]

Several adverse prenatal environmental exposures have already been identified in this context, for example active and passive cigarette smoke [6], and exposure to ambient air pollution [including nitrogen dioxide (NO_2) [7], polycyclic aromatic hydrocarbons (PAH) [8], and particulate matter (PM) [9]]. Particles with a diameter smaller than 500 nm are known to pass the placental barrier during the gestational period, while particles with a diameter smaller than 240 nm are even able to reach the fetal bloodstream, possibly affecting the newborn's metabolism before birth.[10]

2. Ambient air pollution

Since the industrial revolution, levels of ambient air pollution have increased globally. Over the past decades, the effects of these aerial exposures have become increasingly apparent. A first indication of air pollution and its associated effects was noted for the "Great London Smog" in 1952. During this five-day period, the area of and around London was exposed to a suffocating layer of smoke, resulting in the death of an estimated number of 4,000 to 12,000 people.[11] Since then, much attention was brought to the composition of this fog and other air pollution-related exposures, to deduct the effects that could be attributed to the underlying compounds.[12] To date, we see various examples replicating this situation on a global level, due to both man-made and natural sources. Apart from thick layers of smog air pollution in highly populated, industrial areas in China [13] and India [14], one of the most recent examples is the great smog engulfing great areas of Australia and even New Zealand because of the Australian forest fires raging in December 2019. These bushfires could very probably result in an increase in overall mortality and more specifically cardiovascular and respiratory mortality, as could be deducted from studying previous similar disastrous conditions around the area of Sydney.[15] Another very recent example on the effects of air pollution exposure on mortality, is the finding that chances of dying of a COVID-19 infection increase with higher levels of ambient air pollution exposure during the infection period.[16]

2.1. European and global legislation

Both the European Union and the World Health Organization (WHO) have put in place specific guidelines for the average annual exposure levels of several air pollution components. The Air Quality Guidelines for particulate matter (PM), nitrogen dioxide (NO_2), sulphur dioxide (SO_2), and ozone (O_3) of the WHO were updated in 2005 and are based on the summary of scientific evidence on the detrimental effects of air pollution that was available at the time. For PM with a diameter smaller than 2.5 µm ($PM_{2.5}$) the average annual exposure level was set at 10 µg/m³, while that for PM with a diameter smaller than 10 µm (PM_{10}) was set at 20 µg/m³ (**Table 1**). The average annual exposure value for NO_2

determined by the WHO is set even higher, at 40 µg/m³. For O_3 exposure, only an 8-hour mean limit was set in place at 100 µg/m³.

European legislation on Air Quality Standards is less strict than that of the WHO. The average annual exposure levels of both $PM_{2.5}$ and PM_{10} are higher than their respective value of the WHO, put at 25 µg/m³ and 40 µg/m³, respectively. The European value for NO_2 exposure is set equal to that of the WHO, at an average annual exposure of 40 µg/m³ (**Table 1**). Although these EU reference values have not been adapted since their introduction in 2008, a Commission Directive of 2015 has defined several rules concerning the assessment of ambient air quality, with a specification on reference methods, data validation guidelines and sampling locations.[17]

Table 1. Guidelines of the EU and WHO regarding average annual air pollution exposure.

Pollutant	EU guideline	WHO guideline
PM_{10}	40 µg/m³	20 µg/m³
$PM_{2.5}$	25 µg/m³	10 µg/m³
NO_2	40 µg/m³ (= 0.02 ppm)	40 µg/m³ (= 0.02 ppm)

Abbreviations: NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; PM_{10}, Particulate matter with a diameter smaller than 10 µm;

2.2. Origin and composition of ambient air pollution

Air pollution is a complex mixture of substances that can originate from both natural sources, such as forest fires or volcanic eruptions, and anthropogenic sources, such as agriculture, traffic and industrial pollution. The mixture can contain both gaseous components, such as O_3, nitrogen oxides (NO_x) and SO_2, and solid components such as sand, fungal spores and dust particles. Components from an air pollution entity can be both from a primary origin such as ash particles

from fires or volcanic events, and from secondary formation processes, which occur during chemical reactions. Examples of the latter are O_3, SO_2 and NO_2.[18]

Particulate matter (PM) air pollution is composed of a heterogeneous mixture of particles, suspended in either liquid droplets or in a gaseous formation. In general, PM air pollution is divided in three different subclasses, according to the aerodynamic diameter of the particles. PM_{10} is the 'coarse' fraction of particulate matter with a diameter equal to or smaller than 10 μm, while $PM_{2.5}$ encompasses the 'fine' particles with a diameter equal to or smaller than 2.5 μm. The third subdivision is the class of ultrafine particles (UFPs), which entails particles with a diameter of ≤ 0.1 μm. This subdivision not only directs to the size of the particles, but also points out to the clinical effects that the particles can have inside the human body. PM_{10} is retained in the upper part of the respiratory apparatus and can be excreted via retention in the mucus lining. The $PM_{2.5}$ fraction can reach into the smaller branches of the airways and is retained in a substantially larger fraction than PM_{10}.[19] UFP can not only get into the alveoli of the lungs: these small particles can penetrate the air-blood barrier through the alveolar wall and can therefore travel to more distant parts of the body via the bloodstream (**Figure 1**).

2.3. Internal systemic effects

Once PM particles reach the blood stream, several indirect effects of this internal exposure can take place. Various studies in humans and animal models have shown that air pollution exposure is associated with increased levels of inflammatory markers in the bloodstream, such as circulating interleukin 6 (IL-6), and cell surface proteins CD14, CD16, CD4, and CD8.[20] Apart from increased inflammation, elevated exposure to PM air pollution also (co-)induces increased levels of biomarkers associated with endothelial damage [20,21] and platelet coagulation.[22] These systemic events are known to potentially lead to inflammation-related diseases, such as atherosclerosis, and conditions leading to cognitive impairment such as Parkinson's disease and Alzheimer's disease.[22,23]

Another indirect effect of internal air pollution exposure is the increased production of reactive oxygen species (ROS) due to a shift of the tissue's redox system towards oxidative stress. Potential mechanisms of PM-induced oxidative stress include an increase in superoxide ($O_2^{-\bullet}$) production by an elevated expression of superoxide dismutase (24), and oxidative reactions at the surface of the particles were PAHs have been absorbed.(25) Even at the level of the placenta, increased levels of 3-nitrotyrosine (3-NTP), as an indication of nitrosative stress, have been measured in relation with an increment of prenatal exposure to $PM_{2.5}$ and black carbon (BC).[26] In turn, increased oxidative stress responses have been linked with markers of general DNA damage [27] and mitochondrial DNA (mtDNA) damage, such as mitochondrial 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels.[28] Moreover, oxidative stress is known to impact gene expression, such as an elevated expression of transient receptor potential channel 6 (TRPC6), with a higher Ca^{2+} influx as subsequent result.[29]

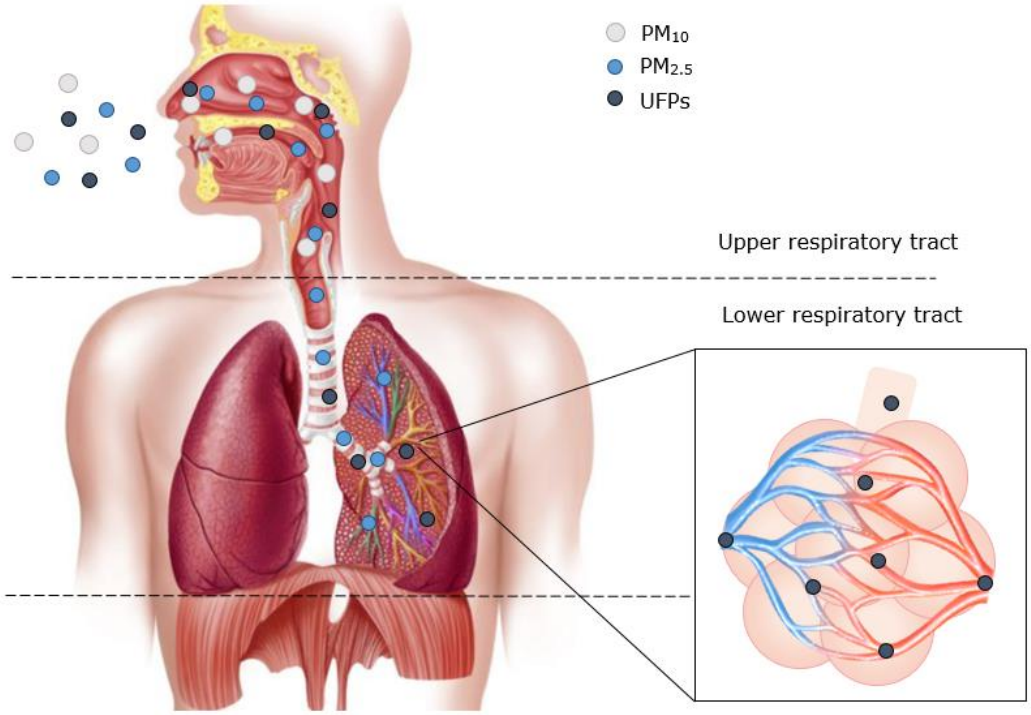

Figure 1. The position of the different particle sizes of air pollution in the respiratory tract. PM_{10} particles (light grey) can only get into the upper part of the respiratory tract, while ultrafine particles (UFPs) (dark blue) can breach the air-blood barrier in the alveoli. *Adapted from https://www.thinglink.com/en-us/scene/1118937718160621569*

2.4. Air pollution exposure and disease development

Exposure to ambient air pollution has been linked to the development of chronic diseases throughout life (**Figure 2**). For example, the increased prevalence of neurodegenerative diseases has been attributed to increased levels of outdoor ambient air pollution, potentially through processes of neuroinflammation and oxidative stress.[23] Furthermore, air pollution exposure has been associated with a higher occurrence of cerebrovascular and cardiovascular disease. A recent study by Fiorito and colleagues emphasizes the link between air pollution exposure and cardiovascular disease with a mediating effect of increased inflammatory and oxidative stress markers.[30] Not only chronic disease incidence, but also overall mortality has been linked to air pollution exposure. Associations with mortality have been found for air pollution in general [31], but also in the context of separate pollution components, such as NO_2 [32] and $PM_{2.5}$.[33]

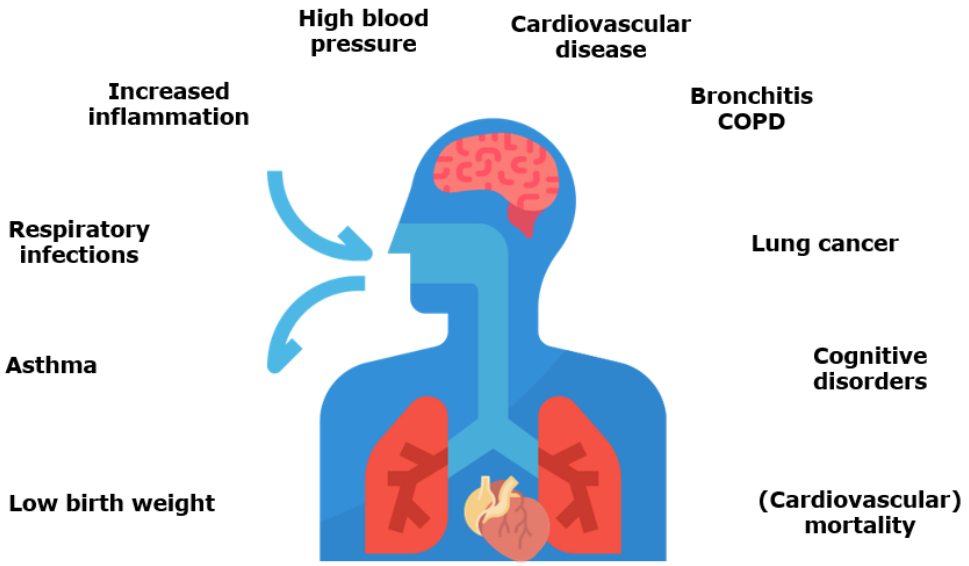

Figure 2. Diseases associated with increased exposure to ambient air pollution throughout life. *Based on the figure printed by the Ozone Basic information. US EPA/ Particulate Matter. Basis Information. U.S. EPA. The BREATHE Project.*

Recently, the role of air pollution exposure during the pregnancy period in disease development has become more apparent. It is expected that the placenta plays an important role in channelling the effects of PM particles from mother to fetus during one of the most crucial phases of development. Black carbon particles can be found at the fetal side of the placenta as early as twelve weeks of gestation.[34] Particles that pass from the maternal circulation through the complex composition of chorionic villae into the fetal circulation are expected to carry on their effects via both direct and indirect pathways (**Figure 3**). Martens and colleagues have shown that exposure to air pollution before birth could have implications for the biological ageing process, by shortening the telomere length in fetal tissues such as the placenta.[35]

3. The placenta

The fetus is able to come into contact with both the direct and indirect effects of maternal exposure through the maternal blood circulation, which connects to that of the neonate via the placenta. Before the 1960's, it was generally assumed that the placenta was a protective barrier, preventing external toxins to reach the developing fetus. This assumption ended abruptly after the thalidomide crisis in 1961.[36] As thalidomide appeared to be teratogenic, women who were given this drug to prevent pregnancy-related nausea gave birth to neonates with severe malformations of the extremities. This event led to stricter regulations of drug development posed upon by the Food and Drug Administration (FDA).[37]

Over the entire intrauterine period, the placenta plays a crucial role for growth, development, and survival of the fetus.[38] After the syncytiotrophoblast cells of the blastocyst have invaded the uterine wall, the placenta starts to grow with the formation of chorionic villi, that constitute the fetal side of this temporary organ (**Figure 3**). One of the first functions of placental cells is to suppress the maternal immune system in such a way that the developing embryo is not rejected.[39]

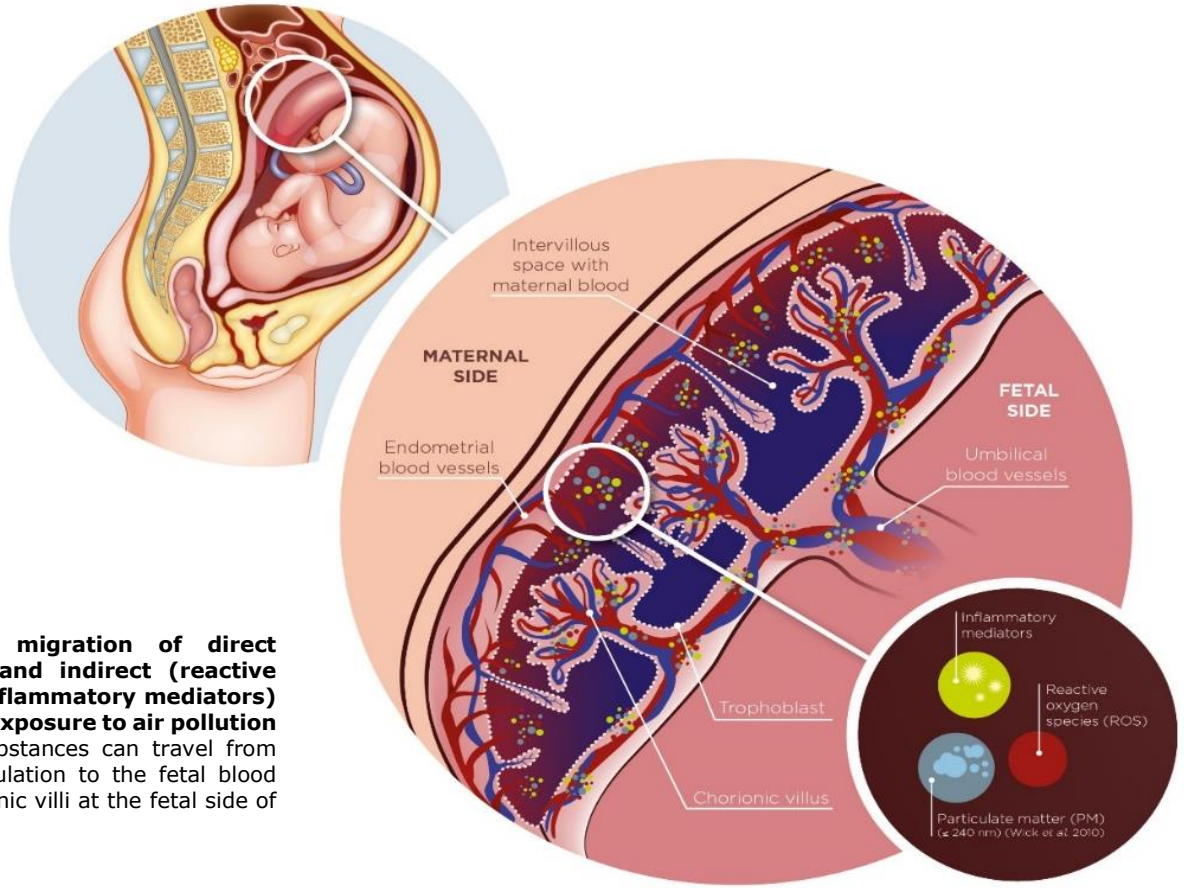

Figure 3. Placental migration of direct (particulate matter) and indirect (reactive oxygen species and inflammatory mediators) potential effectors of exposure to air pollution during pregnancy. Substances can travel from the maternal blood circulation to the fetal blood circulation via the chorionic villi at the fetal side of the placenta.

In later stages of pregnancy, the placenta develops a wide spectrum of functions to ensure proper fetal growth. It is endowed with an important transport function mediating the transfer of oxygen, nutritional components, growth factors, and hormones from mother to child, while carbon dioxide and other waste substances are transferred in the opposite direction.[40] This may occur by means of simple diffusion, (energy driven) transporter proteins, and endo- or exocytosis within complex matrices of different cell types, such as trophoblasts, amniotic cells, endothelium lining of the placental blood vessels, decidual cells, Hofbauer cells, and mesenchymal cells.[38]

In this way, the placenta comes in contact with, contains and interacts with the substances to which both mother and fetus are exposed to during the timespan of the entire pregnancy. In addition, the placenta itself is an important endocrine organ regulating the production of hormones such as progesterone, human chorionic gonadotrophin (hCG), and human placental lactogen (hPL), to ensure the continuation of pregnancy and to acquire the appropriate maternal responses to optimize the development of the fetus.[38,39] Furthermore, within the feto-placental unit, a great number of signals are sent from the placenta to the fetus - and vice versa - to regulate developmental processes.[41] Such signals can also elicit the appropriate reactions to various environmental exposures. Together, all these properties make the placenta an essential organ for the regulation of fetal development. Indeed, placental dysfunction has been linked to for example the occurrence of preeclampsia and adverse birth outcomes such as intrauterine growth restriction.[42]

3.1. Placental tissue in epidemiological research: advantages and disadvantages

Over the past few years, placental tissue has been used as a biological matrix for various epidemiological research purposes. This temporary organ has the advantage that it can serve to evaluate biological outcomes of environmental exposures simultaneously in tissue with both maternal and fetal origin. Moreover, the postpartum sampling of placental tissue requires no invasive procedure, avoiding unnecessary potential damage to the fetus. The placenta shows to be a

crucial tissue to study certain developmental processes, since it provides the necessary molecules for these mechanisms. In mice, it has been shown that this organ produces serotonin at the earliest phases of pregnancy, which is an important factor in the development of the fetal central nervous system.[43] Apart from the different functions of umbilical cord blood and the placenta during pregnancy, several molecular differences between both matrices have been identified such as different turnover rates of mitochondrial DNA (mtDNA).[44] In contrast to cord blood, which can encompass the effects of environmental exposures on the short term, the placenta can reflect the cumulative effect of prenatal exposures over the pregnancy period. In the context of the evaluation of exposure conditions on fetal development, biomolecular measurements in placental samples can be particularly useful since it has been suggested that changes in the placenta could be involved in the epigenetic regulation of fetal development, possibly to a slightly greater extent than in cord blood.[45]

4. Placental -omics signatures of prenatal air pollution exposure

At delivery, the placenta is a representative source of the morphological, functional, biological, and molecular information that has been accumulated during gestation. Therefore, it is a suitable matrix for postnatal investigation of potential associations between molecular (-omics) signatures and prenatal environmental influences. Several biomolecular characteristics related to diverse toxicological exposures have already been investigated in placental tissue. Not only direct DNA damage, but also changes in -omics (genomics, epigenetics, transcriptomics, proteomics, metabolomics and exposomics) signatures can occur due to hazardous environmental exposures such as ambient air pollution (see **Table 2** and **Appendix A** of the manuscript for the complete systematic review on this topic). These alterations could provide early effect predictors for human health risk due to *in utero* environmental exposures.[46]

Table 2. -Omics categories and placental markers analyzed in association with exposure to ambient air pollution during the gestational period

-Omics category	Placental markers
Genomics	- Telomere length [47] - Mitochondrial DNA content [44,48] - Presence of the low activity *EPHX1* (His/His) diplotype [49] - Presence of the *CYP1A1* MspI polymorphism [50] - DNA adduct levels [50–58]
Epigenetics	- Global DNA methylation level [59] - LINE-1 and AluYb8 DNA methylation levels [60] - Mitochondrial DNA methylation level [61] - *LEP* promoter methylation [62] - Levels of miR-21, miR-146a, miR-222, and miR-20a [63]
Transcriptomics	- Expression levels of *BDNF* [64], *SYN1* [64], and *CYP1A1* [50,65]
Proteomics	- 3-NTp levels [26] - Amount of metallothionein [66] - GST level [51] - Activity of AHH [67], Pyruvate kinase [68], GST [51,69], and ECOD [69]

Abbreviations: 3-NTp, 3-nitrotyrosine; AHH, Aryl hydrocarbon hydroxylase; *BDNF*, Brain-derived neurotrophic factor; *CYP1A1*, Cytochrome (*CYP)* P450 1A1; ECOD, 7-ethoxycoumarin O-deethylase; *EPHX1*, Epoxide hydrolase 1; GST, Glutathione S-transferase; His, Histidine; *LEP*, Leptin; miR, MicroRNA; *SYN1*, Synapsin 1

In this context, characteristic biomolecular signatures measured in humans may be considered biomarkers, which can be a chemical or its metabolite, biomolecules, or the product of an interaction between a substance and a target molecule or cell.[70] The measurement of placental -omics markers can provide useful insights on gestational exposure effects, susceptibility, and disease risk of the neonate.[46,71]

4.1. Placental proteomics and in utero air pollution

Although proteins are the end products of the transcription of genomic sequences, there is no linear relationship between a genome and its resulting proteome because of notably alternative splicing and the production of non-functional proteins.[72] Therefore, it is essential that proteomics signatures are studied as a separate -omics field which can complement the finding in other -omics categories.[72] Proteomic characteristics have been investigated in placental tissue in association with maternal tobacco smoking habits [73] and exposure to lead (Pb) and cadmium (Cd) during pregnancy.[74] In addition to the proteome as a whole, considerable focus has been put on separate proteins that for example play an essential role in the detoxification system of the cell. The activity of placental aryl hydrocarbon hydroxylase (AHH), which is the most important metabolizer of PAHs, was significantly higher in placentas obtained from mothers who lived in an environment exposed to urban air pollution compared to the control group.[67] In a study of Sorkun *et al.* [66], a significant increase in the amount of placental metallothionein (MT) was observed in placentas from mothers living in regions with higher levels of ambient air pollution exposure. Another example is pyruvate kinase, an essential enzyme in the process of glycolysis, which showed a significant increase of activity in placental tissue of women who lived in more polluted areas.[68]

Not only the intact proteins themselves, but also the products of protein degradation or modification can be measured as biomarkers of placental reactions caused by detrimental influences during pregnancy. Protein damage can be caused by processes such as oxidative stress and inflammation. In this context,

the tyrosine groups of proteins can be modified into 3-nitrotyrosine (3-NTp) by peroxinitrite, which is an intermediate of oxidative or nitrosative stress. Recently, a positive association was found between placental 3-NTp levels and both $PM_{2.5}$ exposure and BC exposure [26], which is in line with recent studies in mice.[75]

4.2. Prenatal exposure to ambient air pollution, placental biomarkers and disease development

Until now, only few studies have investigated the link between air pollution exposure, the biomolecular characteristics of the placenta and health conditions that could interfere with human development later in life. These studies focussed more specifically on a decrease in birth weight [55,56,67,76], fetal growth restriction [55,67] or the development of bronchitis in early childhood.[49] This shows that the placenta has the potential to serve as a tissue to study the link between prenatal exposures and the effects on the (mal-)development of children in early life.

Ghosh *et al.* [49] investigated the effect of maternal gestational exposure to air pollution in relation to a specific placental genotype and the development of childhood bronchitis during the first two years of life. A significant relationship was identified between the development of childhood bronchitis and the presence of a low activity epoxide hydrolases (EPHX1) polymorphism in the placenta with increased exposure to PAH and $PM_{2.5}$. These authors were thereby the first to identify a link between prenatal exposure to air pollution, a placental -omics marker and disease development. The only mediation analysis to investigate the triple association between air pollution exposure, placental –omics and disease development was performed with data of the INfancia y Medio Ambiente (INMA) cohort, a prospective birth cohort following more than 4000 children from 7 sites in Spain, from birth until the age of 4.[76] This analysis showed that 10% of the association between a 10 µg/m³ increase in NO_2 exposure during pregnancy and reduced birth weight could be mediated by a decrease in mitochondrial DNA levels.[76]

5. Cardiovascular biomarkers of (prenatal) air pollution

Not only -omics markers have been linked to air pollution exposure and disease development. Various clinical signatures of the effects of air pollution exposure have been investigated in association with disease outcomes throughout life. From a cardiovascular point of view, most of the clinical markers that have been associated with air pollution exposure are macrovascular, and not microvascular, in nature. A recent meta-analysis has summarized the knowledge on hypertention, systolic blood pressure and diastolic blood pressure, and described significant positive associations with exposure to PM_{10}, $PM_{2.5}$, SO_2 and NO_2.[77] Another macrovascular parameter that has been associated with air pollution exposure is the carotid intima-media thickness (CIMT). A meta-analysis by Provost and colleagues showed that a 5 μg/m³ increment in $PM_{2.5}$ exposure was associated with a 12.1 μm increase in the CIMT.[78]

Although macrovascular changes are an important indication of cardiovascular health, the significance of the microvasculature in this regard cannot be underestimated. Apart from the strong correlations that exist between macrovascular and microvascular parameters in adults [79], changes in the microvasculature, for example that of the retina, at an early age can be an indicator of cardiovascular disease development later in life.[80]

5.1. The retina: a window to the brain

Cerebral and retinal vasculature share their origin during fetal development and their drainage pathways. Therefore, the blood vessels of the retina can be regarded as a reflection of the conditions in the brain. From their emerging point at the optic disc, the retinal arterioles and venules start branching to cover the entire surface of the retina (**Figure 4**). Since these blood vessels are easily accessible for examination by means of fundus photography, they are often used as a measure of microvascular health. Retinal microvascular characteristics have been studied in association with various parameters such as age, blood pressure, and birth weight, and with disease outcomes such as diabetes mellitus and atherosclerosis.[81] The link between air pollution exposure and the retinal

microvasculature is only beginning to be established. For example, short-term $PM_{2.5}$ exposure on the playground has recently been linked to retinal vessel width in 10-year-old children.[82] However, whether exposure to air pollution during pregnancy has any effect on retinal vessel characteristics, and whether these changes, if any, could be implied as biomarkers of effect, has never been investigated to date.

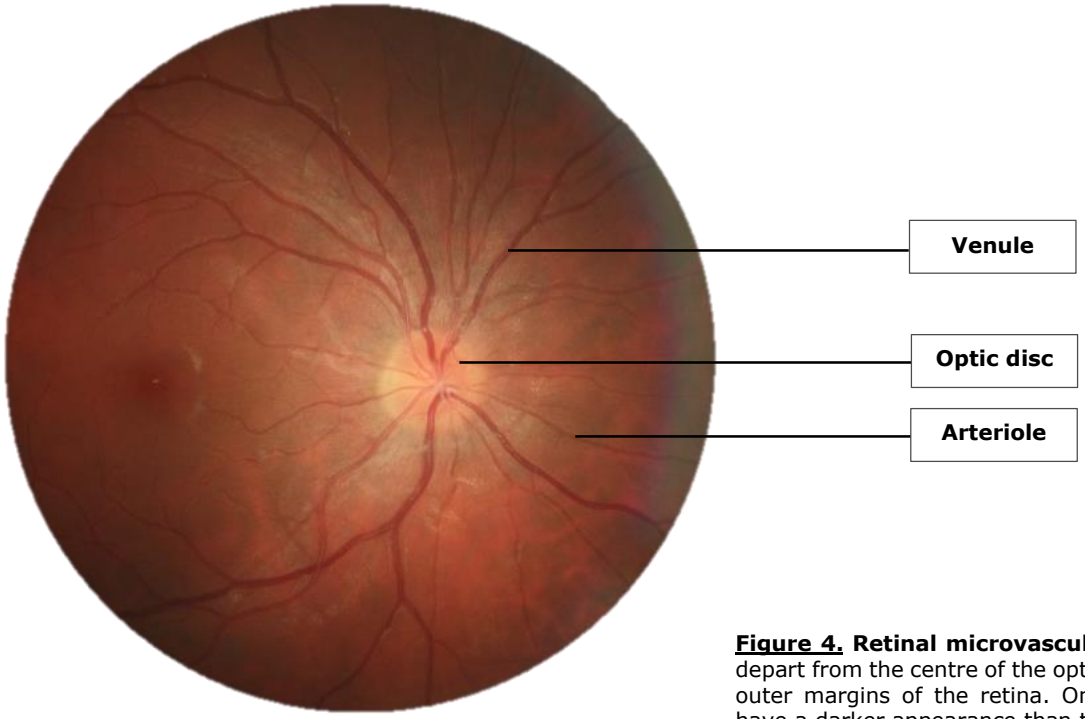

Figure 4. Retinal microvasculature. Arterioles and venules depart from the centre of the optic disc and branch towards the outer margins of the retina. On fundus images, the venules have a darker appearance than the arterioles.

6. Aims of this doctoral dissertation

Although several placental molecular markers of *in utero* air pollution exposure have been identified, most of these studies focused on a bottom-up approach, studying the effects of air pollution exposure on single targets. Furthermore, only few of these studies investigated the postnatal health effects of air pollution exposure during the gestational period, with an apparent lack of information on the effects of exposure on the microvasculature. Therefore, in this doctoral dissertation we studied the molecular effects of prenatal exposure to air pollution on the level of the placental proteome via a top-down approach and examined the effects of prenatal and postnatal exposure to air pollution on a microvascular level.

The specific aims of this work were:

1) **To explore the effects of prenatal exposure to air pollution on the placental proteome**

   In **chapter 1** we described the optimization of a label-free nano-LC mass spectrometric technique to study the placental proteome, with a focus on sample treatment, protein extraction and digestion. In **chapter 2** we used this optimized technique to investigate the effects of differential exposure to ambient black carbon during pregnancy on the placental proteome. Finally, we studied the effects of air pollution exposure during pregnancy on an *a priori* selected target in the placenta, and examined its expression on two expression levels. We selected an important placental TRP channel, TRPC6, and in **chapter 3** we explored the association between prenatal exposure to $PM_{2.5}$ and the expression of this Ca^{2+} transport channel on both the mRNA and protein level.

2) To study the effects of air pollution exposure during pregnancy on the microvasculature at early childhood

To this end, in **chapter 4** we described the effects of both prenatal and postnatal exposure to $PM_{2.5}$ and NO_2 air pollution on both the width and tortuosity of the retinal microvasculature at the age of 4 to 5 years. In the **final chapter** of this dissertation we further investigated whether these microvascular changes reflected the neurological development in this group of children. For this purpose, we analyzed the associations between retinal vessel width and tortuosity, and the outcomes of the cognitive *Cambridge Neuropsychological Test Automated Battery* (CANTAB).

PART 1: PLACENTAL MOLECULAR EFFECTS OF PRENATAL AIR POLLUTION EXPOSURE

CHAPTER 1

OPTIMIZATION OF LABEL-FREE NANO-LC-MS/MS ANALYSIS OF THE PLACENTAL PROTEOME

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CHAPTER 1

ABSTRACT

The placenta can be regarded as a mirror of the events to which the fetus is exposed during development. The placental proteome has been studied with several methodologies differing in sample handling, protein extraction, and processing. We optimized a protocol to analyze the placental proteome by means of label-free nano-LC-MS/MS mass spectrometry with regard to sample treatment, protein extraction, and protein digestion, in order to obtain a high protein concentration for identification of a specific protein signature according to the conditions studied. We recommend mechanical tissue disruption, blood removal prior to protein extraction, and FASP-based or in-gel digestion.

INTRODUCTION

The placenta is a complex organ that can be used postnatally to examine the morphological and molecular effects of environmental exposures or disease processes during pregnancy. The placental proteome has been studied for numerous adverse pregnancy conditions, such as preeclampsia [83,84] and in the context of environmental stressors, such as maternal smoking during pregnancy.[73] Several techniques are currently used in an attempt to identify the entire placental proteome or a placental sub-proteome.[85,86] However, the placenta is a complex tissue with various cell types and it has proven to be very challenging to study the low molecular weight, low abundance placental proteins.[86] Hence, optimization of protein processing prior to the final analysis, for example with label-free mass spectrometry, is needed to improve identification. Protocols applied in different research groups vary in the size of the placental biopsies and in the methods to process the protein extracts into peptides. Therefore, more attention is needed to optimize and unify how to handle placental tissue samples before proteins are extracted, the technique to separate and concentrate placental proteins, and the digestion of the protein mixture into peptides, prior to peptide injection in the mass spectrometer.

Two important aspects of proteomics are sensitivity (the ability to detect the protein if it is present) and specificity (the ability to distinguish a specific target protein from other proteins in the sample) [87], with a high risk of different outcomes for the same research question when different protocols are applied. In addition, since protein material can be lost in each step of the label-free nano LC-MS/MS analysis process, from protein extraction until the final injection of the peptide mixture, a generalized protocol should entail a high number of proteins identified. In this study we optimized an approach to study the proteome of the placenta through label-free nano LC-MS/MS, since this technique allows the untargeted identification of a substantial part of the protein entity, with high accuracy and precision. We paid specific attention to sample treatment regarding blood removal, protein extraction, and post-extraction sample processing.

MATERIALS AND METHODS

Sample collection

Placental tissue samples were collected from 10 randomly selected participants of the ongoing ENVIR*ON*AGE (ENVIRonmental influence *ON* AGEing in early life) birth cohort. The study protocol was approved by the ethical committees of Hasselt University and East-Limburg Hospital (EudraCT B37120107805). Before delivery, an informed consent form was signed by the mothers, and placentas were collected within 10 min after birth.[88] The samples were taken at the fetal side of the placenta, as described by Janssen *et al.* [88] and were either directly frozen in liquid nitrogen or rinsed shortly in phosphate-buffered saline (PBS) before freezing. The biopsies were kept at -80°C until protein extraction.

Protein extraction

Proteins were extracted from approximately 200 mg placental tissue in 1.5 ml lysis buffer containing 2 M thiourea, 7 M urea, 2% CHAPS, and 2% dithiothreitol (DTT). Four tissue disruption methods were tested: by using a syringe (VWR, Pennsylvanian USA), by three subsequent 10-sec sonication bursts (UP100H, Hielscher, Teltow, Germany), by mechanical disruption with an Ultra Turrax T8 mixer (IKA, Staufen, Germany) three times during 10 sec, or by crushing the tissue in liquid nitrogen. The lysates were incubated on a shaking plate for 30 min at 1400 rpm and 15°C and subsequently centrifuged for 5 min at 16.000 g. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA).

Trypsin digestion and detergent removal

Protein precipitation and liquid digestion

Protein extracts were treated with the 2-D Clean-up kit (GE Healthcare Life Sciences, Illinois, USA) according to the manufacturers' instructions. The resulting pellet was re-suspended in 0.2% Rapigest (Waters, Massachusetts, USA), incubated at 15°C for 30 min, centrifuged for 5 min at 13,000 g, heated for 5 min at 100°C and finally kept at -80°C. Samples were reduced with 10 mM dithiothreitol (DTT) and shaken at 500 rpm for 45 min at 37°C, then, alkylated

with 40 mM iodoacetamide (IAA) in the dark under the same conditions. After adding 1 mM $CaCl_2$ and trypsin in a ratio of 1/50 w/w, protein digestion occurred overnight, shaking at 300 rpm and 37°C. The reaction was stopped by adding 2% trifluoroacetic acid (TFA; Biosolve) and incubating for 45 min at 300 rpm and 37°C. After 10 min of centrifugation at 13,000 rpm, the supernatant was used for nano LC-MS/MS analysis.

Liquid digestion with the FASP kit

Samples were digested using filter-aided sample preparation (FASP) digestion, based on modified protocols of the FASP Protein Digestion kit (Expedeon, San Diego, USA).[89,90] We used 10 µg of protein input, and protein digestion was performed with either 1/20 or 1/50 w/w trypsin, for either 2 hours, 5 hours, or overnight at 37°C and 300 rpm. Following final centrifugation during 10 min at 13,000 g, the digestion was stopped with 2% TFA, after which the peptide mixture was directly analyzed with nano LC-MS/MS.

In-gel digestion

Five µl of loading buffer was added to 20 µg of protein sample. This mixture was heated at 100°C for 5 min, centrifuged during 2 min at 13,000 rpm and subsequently loaded on a 10% polyacrylamide gel (Biorad, California, USA). Following a run of 5 min at 200 V and 400 mA, the gel containing proteins was cut into pieces of ± 5-6 mm^3 and kept in H_2O at -20°C prior to trypsin digestion.

Gel fragments were dehydrated with acetonitrile (ACN), shaking 10 min at 900 rpm. After ACN removal, reduction and alkylation of the in-gel protein samples were performed similar to that of the liquid digestion protocol, by incubation with 10 mM DTT and 55 mM IAA. Following two incubation steps with ACN and one with 100 mM NH_4HCO_3, the in-gel protein mixtures were digested with trypsin at a final concentration of 6.25 ng/µl, overnight at 37°C and 300 rpm.

Nano LC-MS/MS

We analyzed the peptide samples with a nano-liquid chromatography Ultimate 3000 system (Thermo Scientific), connected to a maXis Impact electrospray Ultra-High resolution Q-TOF mass spectrometer (Bruker, Massachusetts, USA). Initial

peptide separation occurred by reverse-phase liquid chromatography on a 75 µm by 250 mm C18 column (Acclaim™ PepMap™ 100 C18 LC Column, Thermo Scientific). Mobile phase A in this column was composed of 0.1% formic acid and 2% acetonitrile, mobile phase B contained 0.1% formic acid in 80% acetonitrile. For each run, 2 µg of sample was injected, and the organic concentration of the mobile phase was increased linearly from 4% to 35%. We used a 90 min gradient for in-gel digestion and one of 215 min for other strategies. Subsequently, the resulting flow-through was ionized in the electrospray ionization (ESI) CaptiveSpray (Bruker), which was directly coupled to the C18 column. In survey scan, MS spectra were acquired for 0.5 s in a 50 to 2200 m/z range.

Peak lists were created using the DataAnalysis 4.0 software (Bruker) and uploaded to the ProteinScape 3.1 software (Bruker) which uses Mascot 2.4 (Matrix Science) as protein identifier search engine, through the UNIPROT database (version September 2019, Uniprot-HomoIsoform 190904). Finally, we used the Scaffold 4.8 software (Proteome Software Inc., Portland, Oregon, USA) to visualize the protein identification lists. For the total number of identified proteins, we set the protein and peptide threshold at 1.0% false discovery rate (FDR), and the minimum number of peptides for identification at two. We used the total spectral count method, counting the total number of analysed spectra associated to a single protein group, including those shared with other proteins.

Data analyses and publication

We used the built-in statistical package of the Scaffold 4.8 software (Proteome Software Inc.) for initial protein identification data analyses. Relative protein abundance was determined using the spectral counts method.

We determined the difference in total protein numbers between liquid digestion and FASP-based digestion, and between one and two PBS washing steps, by a two-sided t-test with SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE [91] partner repository with the dataset identifiers PXD020438 and 10.6019/PXD020438.

RESULTS

Mechanical tissue disruption and extra washing improves protein identification

We tested placental tissue disruption with a syringe, sonication, liquid nitrogen, or mechanical disruption. The total number of identified proteins was the highest after mechanical separation (n = 1458) (**Figure 1A**), although the number for sonication did not seem to differ greatly (n = 1330). Because of the presence of more uniquely identified proteins and higher unique peptide counts for important functional proteins (such as ATP synthase and superoxide dismutase) in mechanically disrupted samples compared to those treated with sonication, for all further optimization tests on pre-extraction blood removal and on protein digestion methods, tissue samples were mechanically disrupted.

Using the same biological sample, we performed either no sample treatment, one PBS washing step after tissue collection, or a washing step with PBS both after tissue collection and after thawing to determine the effect on protein identification. PBS washing increased the number of protein identifications (**Figure 1B**). Repeating this experiment, with the conditions of one and two PBS washes in triplicate, showed that after two PBS washes the average total amount of identified proteins increased from 713 to 803, although the difference was not statistically significant ($p = 0.27$) (**Figure 1C**) and that the numbers of several blood-specific proteins decreased (data not shown).

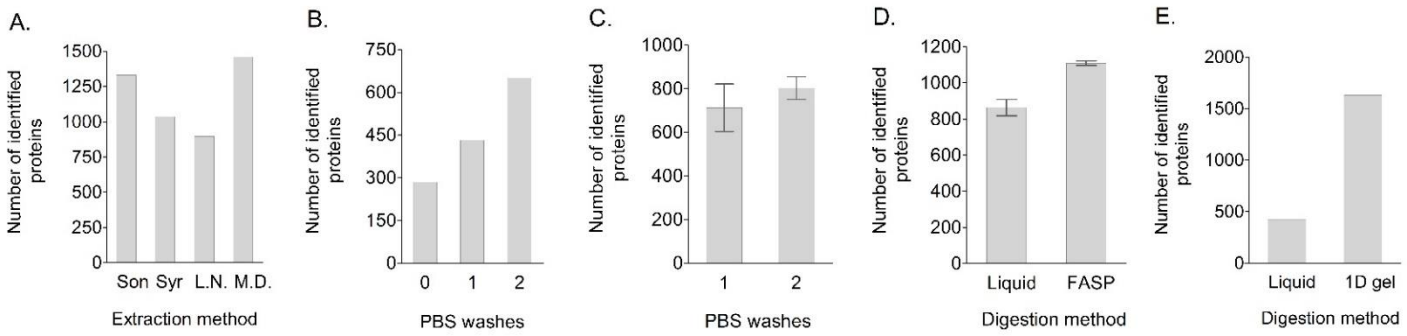

Figure 1. Increase in the total number of identified proteins after protein extraction through mechanical tissue disruption, after a PBS washing step both before freezing and after thawing the samples, and either FASP-system-based or gel-based protein digestion. Figure A: Tested methods of sample tissue disruption are depicted in the x-axis. Mechanical disruption of the placental tissue resulted in the highest number of identified proteins**. Figures B and C:** The x-axis indicates the number of times that the samples had been washed before protein extraction. An extra wash of the placental biopsies after thawing increased the total number of identified proteins. **Figure D:** Protein digestion with the FASP system increased the total number of identified proteins, compared to the numbers obtained with liquid protein digestion. **Figure E:** In-gel digestion following 1D gel sample separation increased the total number of identified proteins, compared to the numbers obtained with liquid protein digestion.

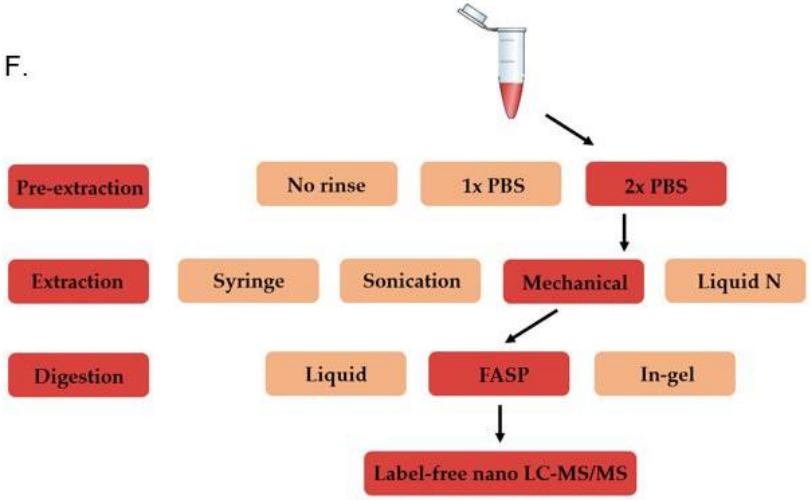

Figure 1. Continued. Figure F: To increase the total number of proteins identified by label-free nano LC-MS/MS, we advise to wash placental tissue samples twice (once directly after taking the biopsies, and a second time before protein extraction) with a neutral substance such as PBS for excess blood removal prior to tissue disruption (line "Pre-extraction"), mechanically disrupt the washed tissue samples (line "Extraction") for protein extraction, and perform FASP-based protein digestion (line "Digestion") for five hours with 1/20 w/w trypsin to minimize the number of miscleavages and non-specific cleavages. Abbreviations: FASP, filter-aided sample preparation; L. N, crushing tissue that was snap-frozen in liquid nitrogen; M. D., mechanical tissue disruption; PBS, phosphate-buffered saline; Son, sonication bursts; Syr, disruption of placental tissue through a syringe.

Increased protein identification with gel- and FASP-based separation and digestion compared to liquid digestion

Firstly, we tested FASP digestion as an alternative for protein precipitation and liquid digestion. We used one biological sample and tested each technique in triplicate. At this optimization stage, all tissue samples were washed both before freezing and after thawing, and were mechanically disrupted for protein extraction. FASP digestion resulted in a higher number of protein identifications (n = 1110) compared to liquid digestion (n = 864) (p = 0.003) (**Figure 1D**). Furthermore, we compared the percentage of non-specific cleavages (i.e. peptides were cut at amino acids other than lysine or arginine) and miscleavages (i.e. trypsin failed to cut the carboxyl end of lysine or arginine). The percentage of miscleavages was approximately the same for liquid- (9.8%) and FASP-based digestion (10.2%), while the percentage of non-specific cleavages was higher for liquid digestion (9.1% vs 3.7%).

Secondly, we compared liquid-based digestion with in-gel digestion of the same biological replicate. Gel-based digestion increased the number of protein identifications (n = 1640) compared to liquid digestion (n = 462) (**Figure 1E**). Although the number of non-specific cleavages was comparable (7.3% for liquid digestion and 5.6% for gel-based digestion), the percentage of miscleavages was higher for gel-based digestion (5.1% vs 17.1%).

In a third test, we compared three digestion periods, i.e. 2 hours, 5 hours, or overnight, in combination with either 1/20 or 1/50 w/w of trypsin. All samples, derived from one biological replicate, were digested by the FASP method. Although the total number of proteins did not differ substantially, the number of miscleavages was higher for the lower trypsin concentrations during each of the three digestions periods (**Table 1**). Overnight digestion increased the number of miscleavages, especially for the digestion with 1/20 w/w trypsin. Therefore, a five-hour digestion with 1/20 w/w trypsin appears to be most favorable.

Table 1. Total number of protein identifications and percentage of miscleavages and non-specific cleavages for each of six conditions combining three different digestion durations and two trypsin concentrations.

Incubation conditions (duration – w/w trypsin)	Total number of identified proteins	Miscleavages (%)	Non-specific cleavages (%)
2h - 1/50	392	23.0	0.3
2h - 1/20	352	16.0	2.7
5h - 1/50	382	19.6	1.4
5h - 1/20	349	11.9	1.5
ON - 1/50	374	11.3	2.2
ON - 1/20	359	7.2	7.3

Abbreviations: ON, overnight

DISCUSSION

We have evaluated a protocol to analyze the placental proteome through label-free nano-LC-MS/MS. Our key findings are: 1) Blood removal prior to protein extraction and mechanical tissue disruption increase the total amount of identifications; 2) In-gel and FASP-based protein digestion result in a higher number of identified proteins, compared to liquid-based digestion and protein precipitation, and 3) To reduce the number of miscleavages and non-specific cleavages during placental protein digestion, a concentration of 1/50 w/w trypsin and overnight digestion are not advised (**Figure 1F**). Depending on the research question and number of samples, research groups can choose between FASP-based digestion or in-gel protein digestion, of which the latter is more labour-intensive and time consuming (due to all gel fragments for 1 sample being analysed separately), but results in higher protein identification compared to FASP-based digestion.

One of the challenges of using placental tissue in proteomic research is its complexity and heterogeneity. Concerning cellular compartment distribution, our placental protein extracts contained a higher percentage of mitochondrial proteins, and a similar number of cytosol- and membrane-related proteins for all

extraction techniques in comparison with the human proteome.[92–94] Nuclear proteins could be identified to a lower level compared with the human proteome for each technique, apart from the technique without blood removal before protein extraction, for which no nucleus-related proteins were found (**Supplemental Table 1**).

Recent research suggests that the placenta contains different sub-proteomes, rather than one proteome entity.[95] Therefore, tissue sampling is an essential initial step in placental proteomic research. Burton and colleagues have optimized a protocol regarding the placental storage and sampling process for various purposes.[96] If these steps proceeding sample treatment and protein digestion would be combined and unified for proteomic research purposes in placental tissue, this would aid reproducibility and reliability in the search for protein biomarkers related to the impact of environmental exposure or disease development during pregnancy.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Percentage of proteins that can be subdivided into the GO cellular location category for mitochondrial proteins, nuclear proteins, proteins related to the plasma membrane, and cytosol proteins.

Location	0 washes	1 wash	2 washes	Sonication	Syringe	Liquid nitrogen	Mechanical disruption	Liquid digestion	FASP digestion	1D-gel digestion
Mitochondria	15.8	16.1	18.6	15.3	15.9	18.4	18.4	13.6	17.8	16.1
Nucleoplasm	0	3.3	3.8	2.8	2.7	6.7	3.3	6.7	1.7	12.6
Membrane	30.1	28.0	30.6	25.7	26.8	25.2	25.9	24.4	26.3	27.2
Cytosol	43.1	38.4	31.5	32.6	31.6	33.6	30.3	31.7	34.0	34.2

Classification of the protein lists was determined with an enrichment analysis through the online DAVID classification system (version 6.8), and its results obtained from the Gene Ontology (GO) cellular component database. Percentages were calculated as the number of proteins identified for each category divided by the total amount of identified proteins for each technique. Abbreviations: FASP, Filter-aided sample preparation.

CHAPTER 2

ALTERATIONS IN THE PLACENTAL PROTEOME IN ASSOCIATION WITH THE PLACENTAL BLACK CARBON LOAD DURING GESTATION

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In preparation

ABSTRACT

Background: Exposure to ambient air pollution is known to cause direct and indirect molecular expression changes in the placenta, on the DNA, mRNA and protein level. Ambient black carbon (BC) particles can be found in the human placenta already very early in gestation. However, the effect of *in utero* black carbon exposure on the entire placental proteome has never been studied to date.

Objectives: We explored whether expression of the placental proteome differs between mothers exposed to either high or low levels of BC throughout the entire pregnancy period.

Methods: We used placental tissue samples from the ENVIR*ON*AGE birth cohort, of 20 non-smoking, age- and neonate characteristic-matched women exposed to either high (n = 10) or low (n = 10) levels of BC throughout pregnancy. We modeled prenatal BC exposure levels based on the mother's home address and measured BC levels in the fetal side of the placenta. The placental proteome was analysed by nano-liquid chromatography Q-TOF mass spectrometry and we used PEAKS software for protein identification and label-free quantification. Protein-protein interaction and functional pathway enrichment analyses were performed with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online software.

Results: Thirteen proteins showed an at least 2-fold expression difference between the 2 exposure groups, all overexpressed in the placentas of women exposed to high levels of BC during pregnancy. Three protein-protein interactions were enriched within this group, namely between TIMP3 and COL4A2, SERPINE2 and COL4A2, and SERPINE2 and GP1BB. Functional pathway enrichment analysis put forward pathways involved in extracellular matrix receptor interaction, fibrin clot formation and sodium ion transport regulation.

Conclusions: Prenatal black carbon exposure affects the proteome of the placenta. Future research should focus on the potential consequences of these alterations on both placental functioning, and health and disease during early childhood development.

INTRODUCTION

The effect of ambient air pollution exposure on placental protein expression has not been studied to a great extent.[97] Only several pre-selected targets have been linked with exposure to air pollution during pregnancy, with the largest focus on proteins with a role in the cellular detoxification system.[51,67,69] Recently, black carbon (BC) particles have been detected in the fetal side of the human placenta, as early as 12 weeks of gestation.[34] The quantifty of particles in placental tissue was strongly and positively associated with the ambient BC levels to which the mother had been exposed throughout pregnancy. *In utero* black carbon exposure has been recently associated with increased mitochondrial methylation levels [98], an elevated Alu mutation rate (a marker of general DNA mutation) and an increased promoter methylation in APEX1 and ERCC4.[99] In addition, prenatal air pollution has been associated with shorter telomere length, a marker of ageing, at birth.[35] However, to our knowledge, the association between *in utero* exposure to ambient air pollution such as black carbon and the induced changes on a proteome-wide level have never been examined. The effects of black carbon exposure on the proteome has been studied in several (non-placental) cell line and animal models. In the human lung epithelial cell A549 model, BC upregulated 9 specific proteins compared to for example TiO_2 exposure, related to cell proliferation and apoptosis.[100] Label-free nano LC-MS/MS analyses of bronchoalveolar lavage fluid in BC-exposed mice revealed a pattern of specific up- and downregulated proteins related to inflammation and surfactant production.[101]

In this study, we have examined the effects of exposure to ambient BC air pollution during pregnancy on the placental proteome at birth. For this purpose, we compared placental tissue samples of the ENVIR*ON*AGE prospective birth cohort of women exposed to either high or low levels of BC, and analysed the protein composition by means of label-free nano LC-MS/MS analysis.

MATERIALS AND METHODS

Recruitment and eligibility

This study population is part of the ongoing prospective ENVIR*ON*AGE (ENVIRonmental influence *ON* AGEing in early life) birth cohort.[88] The study was conducted according to the guidelines described in the Declaration of Helsinki, and the study protocol was approved by the ethics committees of the East-Limburg Hospital (Genk, Belgium) and Hasselt University (Diepenbeek, Belgium). The twenty mothers of whom the samples were used in this project were recruited at the maternity ward of the East-Limburg Hospital between 12 am on Fridays and 7 am the subsequent Mondays, between the 18th of February 2012 and the 6th of October 2016. All participants signed an informed consent document before giving birth.

Women were eligible for participation if they carried a singleton pregnancy and were able to fill in a Dutch questionnaire. This questionnaire, filled in after delivery, provided us details on both clinical information, such as the mothers' age and pre-pregnancy body mass index (BMI), and life style information, such as her educational level, smoking habits and residential address(es) throughout the entire pregnancy. The neonate's date of birth, gestational age, sex, birth weight, and birth length were obtained from its medical files. Maternal education, as a proxy for socio-economic status, was categorized as low (no diploma or primary school), middle (high school diploma), and high (college or university degree). Since (e-)cigarette smoke is a source of black carbon [102], we selected maternal smoking during pregnancy as an exclusion criterium for this study.

Exposure assessment

In our cohort, we were able to assess both ambient BC exposure levels and BC particle load at the fetal side of the placenta. Exposure to ambient BC during pregnancy was determined by the mothers' residential address, by means of the Kriging spatial-temporal interpolation method.[103] This method combines a dispersion model with air pollution exposure data provided by official fixed monitoring stations in Flanders, and land cover data obtained from satellite images of the CORINE land cover dataset.[104,105] This model chain provides interpolated air pollution values from the Belgian telemetric air quality networks

on a dense, irregular high-resolution receptor point grid from both point sources (such as industrial sites), and line sources (such as highways or major roads). The overall model performance was assessed by leave-one-out cross-validation, including 16 monitoring stations for BC. Validation statistics determined a spatiotemporal explained variance of more than 0.74. Moreover, the accuracy of the exposure models was recently demonstrated by the association between the urinary load of nano-sized black carbon particles in children and their residential BC levels.[82]

Placental tissue black carbon load was determined by means of non-incandescence-related white-light generation of the BC particles under femtosecond illumination, as described previously.[34,106] Placental samples were fixed in formaldehyde for at least 24 hours and embedded in paraffin. Tissue sections of 4 µm were obtained with a microtome (Leica Microsystems, UK). To avoid ambient BC particulate contamination, sections were made in a clean room with filtered air (Genano 310/OY, Finland) using particle-free instruments and sample holders.

Images of the placental fractions were obtained with a Zeiss LSM 510 confocal laser scanner (Carl Zeiss, Jena, Germany) containing a two-photon femtosecond pulsed laser (MaiTai DeepSee, Spectra-Physics, USA). The scanner was pre-set to a central wave length of 810 nm, on average 5 or 10 mW radiant power, with a 10×/0.3 objective. White-light emission produced by the BC particles was obtained with two filters, between 400–410 nm and 450–650 nm, to image both two-photon excited autofluorescence of the placental cells and second harmonic generation from collagen type I, respectively. A tile scan of 100 images was acquired within a field view of 9000 × 9000 μm^2 with a pixel size of 0.694 µm and a pixel dwell time of 2.51 µs. The number of BC particles in the scans of each placental section was determined with an automated and customized Matlab program (Matlab 2010, Mathworks, The Netherlands). The BC load was measured in three regions of each placental sample, within five different sections taken in the middle of the biopsy (n = 15 images).

To determine the duration of the entire pregnancy period, the conception date was estimated by the first day of the mother's last menstrual period and

ultrasound imaging data.[44] For the two participants who moved during pregnancy, we calculated and combined the exposures for both residential addresses, thus taking into account the changes in exposure during the corresponding period. High prenatal residential BC exposure was defined as having both an entire pregnancy and third trimester residential BC exposure ≥ 75th percentile (1.70 μg/m³ and 2.42 μg/m³, respectively), and a residential proximity to a major road ≤ 500 m. Low prenatal residential BC exposure was defined as having both an entire pregnancy and third trimester residential BC exposure ≤ 25th percentile (0.96 μg/m³ and 0.63 μg/m³, respectively), and a residential proximity to a major road > 500 m.[34]

Placental tissue sampling, protein extraction and protein digestion

Placentas were collected and deep-frozen within 10 min after child birth. Tissue samples were taken according to a standardized method at four fixed sites on the fetal side of the placenta, four cm from the center of the umbilical cord. The position of the sites was determined clockwise by the largest placental vein. Samples were taken 1.0 cm to 1.5 cm beneath the chorio-amniotic membrane and were snap-frozen immediately and kept at -80°C until protein extraction.

Proteins were extracted from a pooled sample of all four quadrants of each placenta, with a final weight of 40 mg. The below described protocol for sample treatment, protein extraction and peptide digestion was performed as optimized previously.[107] All pooled samples were thoroughly washed with phosphate buffered saline (PBS) before freezing and a second time after thawing, and were subsequently put in 300 μL extraction buffer, consisting of 2% CHAPS, 2% DTT, 2 M thiourea and 7 M urea. Tissue samples were disrupted mechanically with an Ultra Turrax T8 mixer (IKA, Staufen, Germany) three times during 10 sec. Subsequently, the lysates were incubated on a shaking plate for 30 min at 1400 rpm and 15°C and subsequently centrifuged for 5 min at 16,000 g. Protein concentration was determined in the resulting supernatant with the Pierce BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA). Extracts were kept at -80°C until protein digestion.

Protein extracts were digested using modified filter-aided sample preparation (FASP) method as described by Wiśniewski *et al.* and Distler *et al*.[89,90]

All centrifugation steps were performed at 11,000 g. In short, 10 µg of protein was added onto the filter, followed by 200 µL of 8 M urea acetate (UA). After 15 min of centrifugation, the protein mixtures were reduced with 8 mM dithiothreitol (DTT) while incubating at 56°C for 15 min. Next, the samples were alkylated with 50 mM iodoacetamide (IAA) during a 20 min incubation at room temperature, and following centrifugation and a washing step with UA, excess IAA was removed by a second incubation with DTT. After three washing steps, one with UA and two with 50 mM NH_4HCO_3, proteins were digested with trypsin (1:50 enzyme to protein) for 5 hours at 300 rpm. Following a final centrifugation during 10 min at 13,000 g, the digestion was stopped by adding 2% trifluoroacetic acid (TFA; Biosolve) and the resulting peptide mixture was analyzed with nano LC-MS/MS.

Nano LC-MS/MS

The digest was analyzed using nano-LC-ESI-MS/MS timsTOF Pro (Bruker, Billerica, MA, USA) coupled with an UHPLC nanoElute (Bruker) for two injections/technical replicated per digest. Peptides were separated by nanoUHPLC (nanoElute, Bruker) on a C18 column (75 µm ID, 25 cm) with integrated CaptiveSpray insert (Aurora, ionopticks, Melbourne) at a flow rate of 400 nl/min and at 50°C. LC mobile phase A contained water with 0.1% formic acid (v/v) and phase B consisted of ACN with 0.1% formic acid (v/v). Samples were loaded directly onto the analytical column at a constant pressure of 800 bar. One microliter of the peptide mixture was injected, and the organic content of the mobile phase was increased linearly from 2% to 15% phase B in 60 min, from 15% to 25% phase B in the next 30 min, from 25% to 37% phase B in the subsequent 10 min and from 37% to 95% phase B in the final 5 min. Data acquisition on the timsTOF Pro was performed using Hystar 5.0 and otof-Control 6.0., using 160 ms TIMS accumulation time, and a mobility (1/K0) range from 0.7 to 1.4 Vs/cm². Mass-spectrometric analysis was carried out using the parallel accumulation serial fragmentation (PASEF) acquisition method.[108] One MS spectrum was followed by six PASEF MSMS spectra per total cycle of 1.16 s.

Data analysis was performed using PEAKS Studio X+ with ion mobility module and Q module for label-free quantification (Bioinformatics Solutions Inc., Waterloo, Canada).[109] Protein identifications were conducted using the PEAKS search engine

with 15 ppm as parent mass error tolerance and 0.05 Da as fragment mass error tolerance. Carbamidomethylation was allowed as fixed modification, oxidation of methionine and acetylation (N-term) as variable modification. Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at one. The peak lists were searched against the Homo Sapiens and isoforms protein database from UNIREF 100 (173190 sequences). Peptide spectrum matches and protein identifications were normalized to less than 1.0% false discovery rate. Label-free quantitation (LFQ) is based on a determination of the area under the curve, and calculates the expectation - maximization algorithm on the extracted ion chromatograms of the three most abundant unique peptides of a protein.[110] For protein quantification, the mass error was set to 20 ppm, ion mobility tolerance to 0.08 1/k0, peptide quality score to ≥ 4 and the protein significance score threshold was set to 20. The significance score was calculated as the -10log10 of a p-value of 0.01. The software used an ANOVA test to determine statistical significance. Modified peptides were excluded and only proteins with at least two identified peptides were used for quantification. Total ion current was used to calculate the normalization factors.

Statistics analysis

We used a two-sided Student's t-test to determine potential differences in variables between both exposure groups. We performed protein quantification, and determined differential expression values between the two groups, by means of PEAKS software. To determine differentially expression proteins, the PEAKS algorithm used a two-way ANOVA, with a set significance of 20 (corresponding to a p-value of ≤ 0.01) and a fold change of ≥2 (and an additional, exploratory analysis with a fold change of ≥1.5) and ≥ 2 peptides per protein used for quantification. Finally, we set in the PEAKS software that a peptide had to be detected in at least 12 samples per group. For all protein groups that passed the former quantification filters, the software used Benjamin–Hochberg multiple testing correction to adjust the p-value for the false discovery rate (FDR) calculation. Only proteins with significance scores passing the adjusted FDR were provided in the final listing.

Protein-protein interaction analysis

For protein-protein interaction analysis, we used the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software (version 11.0). This analysis gives the number of interactions between the entered proteins, and compared these against the number of interactions that would be expected from a random set of proteins. Furthermore, STRING determines the functional pathways and systems that are enriched in a set of proteins, using a combination of several online classification systems such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [111] and Gene Ontology (GO) [112] databases to determine and describe protein-protein interactions in terms of enriched functional associations.[113,114] STRING pathway enrichment calculations apply Benjamini-Hochberg multiple testing correction, within each separate functional classification framework (such as GO and KEGG).[114]

RESULTS

Population and exposure characteristics

The mothers in the low and high exposure group had similar characteristics, as summarized in **Table 1**. On average [standard deviation], mothers gave birth at the age of 29.9 [4.2] years. The education level of the mothers was high [90.0%]. Maternal pre-pregnancy BMI was slightly higher in the low exposure group (29.0 [7.4] kg/m²) compared to the high exposure group (22.4 [3.8] kg/m²). The average ambient black carbon exposure level in the low exposure group was 0.82 [0.09] μg/m³, and 1.94 [0.24] μg/m³ in the high exposure group. Average placental BC load reflected the ambient levels, with a 2-fold higher average in the high exposure group (20943.4 [9642.3] particles/mm³) compared to the low exposure group (9542.7 [6645.7] particles/mm³). The neonates (50.0% girls) had an average gestational age of 39.8 [2.0] weeks, weighed 3395.0 [438.7] g and had a length of 49.9 [2.0] cm. None of these variables showed a significant difference between the high and the low exposure group.

Table 1. Average (SD) or numbers (%) of the characteristics of the participants included in this study (n = 20).

Characteristic	Total population	Low BC exposure	High BC exposure	p-value
Mother				
Age at birth child (years)	29.4 (4.2)	29.2 (3.6)	29.4 (4.9)	0.41
Pre-pregnancy BMI (kg/m²)	25.7 (6.6)	29.0 (7.4)	22.4 (3.8)	0.06
Education level				0.82
Low (no high school diploma)	2 (10.0)	1 (10.0)	1 (10.0)	
Middle (high school diploma)	7 (35.0)	4 (40.0)	3 (30.0)	
High (college degree or higher)	11 (55.0)	5 (50.0)	6 (60.0)	
Prenatal black carbon exposure (µg/m³)	1.38 (0.60)	0.82 (0.09)	1.94 (0.24)	< 0.0001
Child				
Sex				1.00
Girls	10 (50.0)	5 (50.0)	5 (50.0)	
Birth Weight (g)	3395.0 (438.7)	3449.5 (497.8)	3340.5 (389.6)	0.59
Birth Length (cm)	49.9 (2.0)	49.9 (2.6)	50.0 (1.4)	0.94
Gestational age (weeks)	39.8 (1.0)	40.1 (0.9)	39.5 (1.1)	0.19

Abbreviations: BMI, body mass index. Average data are presented with standard deviation (SD).

Protein identification

The results of one subject were excluded in the analyses, due to insufficient quality of the two technical replicates reflected by a substantial difference in protein levels. With two technical replicated analysed for each biological sample, this resulted in a total of 38 samples used for protein identification and quantification. Sample clustering shows that the two technical replicates of each of the 19 remaining biological samples are strongly clustered, indicating low technical variability in protein identification (**Figure 1**). Moreover, a strong clustering was identified between the samples within the same BC exposure group, indicating a great similarity in protein profile within the samples of either the high or low BC group. In the 38 samples combined, 64,373 unique peptides and 8556 unique proteins were identified. Of this total number of unique proteins, 13 proteins were found to have a ≥ 2-fold change difference between the high and low prenatal BC exposure group.

Increased protein expression in high black carbon exposure group

Two-way ANOVA statistical analysis showed that the expression from 13 proteins was significantly 2-fold different between placentas from both exposure groups (**Table 2**). All proteins showed a higher expression in the placentas of women exposed to high levels of prenatal black carbon. Protein-protein interaction (PPI) enrichment analysis showed that between the 13 proteins (nodes) there were 3 significant interactions (edges), while none were expected, with a PPI-enrichment p-value of 0.015 (**Figure 2**). Enriched PPI were identified between metalloproteinase inhibitor 3 (TIMP3) and collagen alpha-2(IV) chain (COL4A2), glia-derived nexin (SERPINE2) and COL4A2, and SERPINE2 and platelet glycoprotein Ib beta chain (GP1BB). Pathway enrichment analysis in STRING put forward the extracellular matrix (ECM)-receptor interaction pathway to be enriched from the KEGG pathway databases, two processes were enriched in the GO databases, namely the regulation of sodium ion transport and intermediate filament organization and two pathways from the Reactome Pathways database were determined to be enriched, related to fibrin clot formation and hemostasis (**Table 3**).

For a more exploratory view on the data, we performed a second functional enrichment analysis in STRING, taking into account proteins that showed at least 1.5-fold difference in expression between the two exposure groups. In total, 59 proteins with a ≥ 1.5-fold change were significantly different between the high and low exposure group, with 41 proteins being overexpressed in the high exposure group, and 18 proteins in the low exposure group (**Figure 2 and Supplemental Table 1**). In the high exposure group, apart from the ECM-receptor interaction pathway to be enriched from KEGG database, seven additional pathways were enriched (**Supplemental Table 2**). In the low exposure group, no KEGG or GO pathways were enriched within the 18 proteins, although there were four significant protein-protein interactions within this group, between CYTC and CDA, GCSH and ALDA4H1, GLRX and IBA57, and between PHF5A and PFDN5.

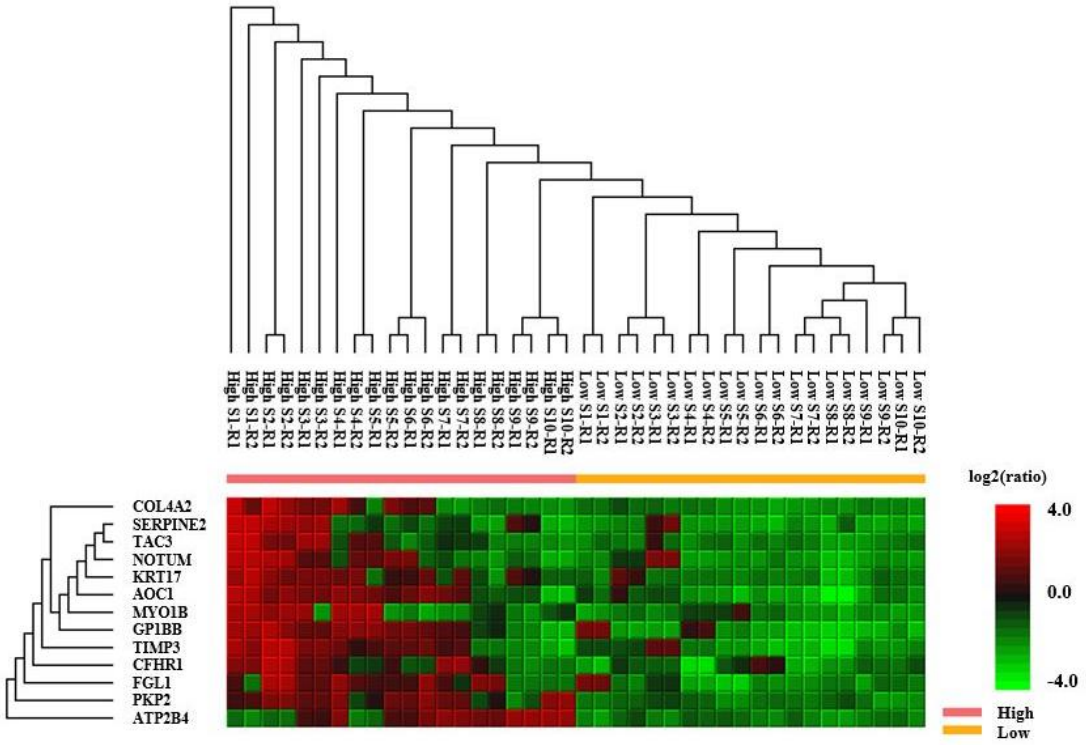
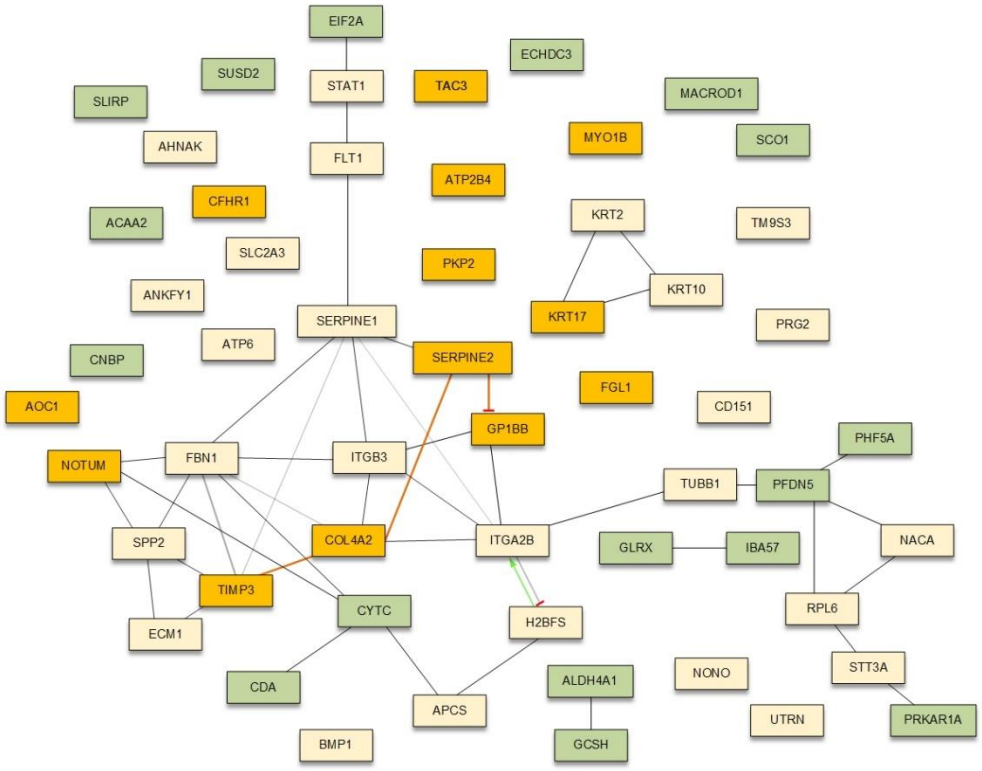


Figure 1. Protein profile heatmap of the 13 proteins with a ≥ 2-fold change in association with black carbon exposure during pregnancy. Cell colour represents the log2(ratio) to the average abundance across different samples. Samples are clustered vertically according to sample similarity, which shows a strong clustering pattern for the technical replicates of the same biological sample (R1 and R2). Protein expression levels are shown as colored boxes; red indicates a high expression level and green indicates a low expression level.



<u>Figure 2.</u> Differentially expressed proteins in the placental tissue of women exposed to high levels of black carbon air pollution during pregnancy. Proteins with an at least 2-fold higher expression are depicted in dark orange, proteins with a fold change difference of 1.5 to 2 are either depicted in light orange in case of overexpression, and in green in case of underexpression. Two overexpressed proteins (identified as "dihydropyrimidinase-like 2" and "cDNA FLJ54854 highly similar to Junctional adhesion molecule A") are not represented in this figure, since no official abbreviation has been determined for these targets. Proteins connected with a line represent the significant protein-protein interactions as analysed with STRING enrichment analysis. Direct inhibitory reactions are shown by a red parallel line, direct activation is depicted with a green arrow.

Table 2. Proteins with a 2-fold higher expression in placental tissue in association with prenatal BC air pollution exposure.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides
plakophilin-2	PKP2	Q99959	73.59	2
metalloproteinase inhibitor 3	TIMP3	P35625	32.53	7
keratin, type I cytoskeletal 17	KRT17	Q04695	48.3	6
complement factor H-related protein 1	CFHR1	Q03591	36.8	12
plasma membrane calcium-transporting ATPase 4	ATP2B4	P23634, A0A024R968	53.4	3
collagen alpha-2(IV) chain	COL4A2	P08572, A0A024RDW8	30.41	2
unconventional myosin-Ib	MYO1B	E9PDF6, O43795, B0I1S9	25.73	3
fibrinogen-like protein 1	FGL1	Q08830	41.96	12
platelet glycoprotein Ib beta chain	GP1BB	P13224, A0A140GX60, A0A140GX63	48.79	3
palmitoleoyl-protein carboxylesterase NOTUM	NOTUM	Q6P988	27.65	2
amiloride-sensitive amine oxidase [copper-containing] 1	AOC1	P19801, D3DX01	43.4	18
glia-derived nexin; Serine protease inhibitor	SERPINE2	P07093, A0A024R451	31.12	10
tachykinin-3	TAC3	Q9UHF0, A0A024RB47	29.32	4

Proteins with a ≥ 2-fold expression difference between the low and high black carbon exposure group are listed with their full name, short name and accession(s) as found in the UniProt database. Additionally, the number of unique proteins identified for each protein is provided, together with the significance score computed by the PEAKS software, as (-10 x log(p-value)).

Table 3. Results of the STRING functional pathway enrichment analysis on placental proteins with a ≥ 2-fold change in association with prenatal black carbon air pollution exposure.

Resource	Pathway ID	Pathway description	Proteins involved	False discovery rate
KEGG	hsa04512	ECM-receptor interaction	COL4A2, GP1BB	0.0376
GO Process	GO:0002028	Regulation of sodium ion transport	ATP2B4, PKP2, SERPINE2	0.0165
GO Process	GO:0045109	Intermediate filament organization	KRT17, PKP2	0.0419
Reactome Pathway	HSA-140877	Formation of Fibrin Clot (Clotting Cascade)	GP1BB, SERPINE2	0.011
Reactome Pathway	HSA-109582	Hemostasis	ATP2B4,GP1BB,SERPINE2,TIMP3	0.0115

Abbreviations: ATP2B4, plasma membrane calcium-transporting ATPase 4; COL4A2, collagen alpha-2(IV) chain; ECM, Extracellular Matrix; GO, Gene ontology; GP1BB, platelet glycoprotein Ib beta chain; KEGG, Kyoto Encyclopedia of Genes and Genomes; KRT17, keratin, type I cytoskeletal 17; PKP2, plakophilin-2; SERPINE2, glia-derived nexin; TIMP3, metalloproteinase inhibitor 3.

DISCUSSION

In this study, we examined the effect of prenatal exposure to BC air pollution on the placental proteome. We found that 13 proteins were ≥ 2-fold upregulated in the placentas of mothers who were highly exposed to black carbon, compared to those of the low exposure group. Protein-protein interaction analysis showed that GP1BB, SERPENTIN2, COL4A2 and TIMP3 had a stronger interaction than expected by chance. Through pathway enrichment analysis, we identified five pathways linked to the ≥ 2-fold upregulated proteins, related to sodium ion transport, fhe formation of fibrin clots, haemostasis, intermediate filament organization and the ECM-receptor interaction pathway, indicating the disruption of these cellular processes in association with elevated levels of prenatal ambient BC exposure.

Thirteen proteins were identified as having an at least 2-fold higher expression, and 59 proteins were differentially expressed if the fold change was set on 1.5 or higher. In a study from Wang and colleagues, comparing the placental proteome of normal and preeclampsia pregnancies, preeclampsia was shown to cause differential expression of 171 placental proteins with at least a 1.5-fold change [84], while another study on preeclampsia using 2DE and MALDI-TOF MS identified 20 differentially expressed spots from placental tissue extracts. When studying direct exposure to black carbon (and many other) particles via cigarette smoke, Huuskonen and colleagues showed by means of 2-D gel separation and spot picking that 18% of the protein spots were differently expressed in mothers who smokes during pregnancy.[73] However, the authors took into account less stringent thresholds: 1.2 times difference, compared to a ≥ 2-fold difference in our study.

The thirteen differentially expressed targets from our analyses were identified only within the high BC exposure group. None of these proteins has ever been directly associated with black carbon or other (prenatal) air pollution exposure, however previous evidence was found on a relation between one of the thirteen identified targets, SERPINE2, with maternal smoking during pregnancy. SERPINE2 has been associated with gestational maternal smoking and childhood pulmonary function in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) cohort, a prospective study containing the genotyping records of 1996 children aged 8

years.[115] Children with SERPINE2 allele SNP rs729631 had a higher forced vital capacity (FVC) suggesting a protective function against the effects on maternal smoking during pregnancy.

One of the enriched protein-protein interactions was that between extracellular matrix proteins COL4A2 and TIMP3. Both COL4A2, one of the subunits of collagen type 4 which forms an important structural component of basement membranes, and TIMP3, an inhibitor of matrix metalloproteinases that break down the ECM, have been found to be overexpressed in the placental tissue of pre-eclampsia pregnancies.[116,117] The increased expression of these genes is thought to contribute to the physiological characteristics of this condition: dysregulated COL4A2 would result in shallow trophoblast invasion and an increased production of the COL4A2 product canstatin, could contribute to anti-angiogenic effects such as increased apoptosis and endothelial dysfunction.[117,118] Increased expression of TIMP3, in preeclamptic conditions potentially regulated by promotor hypomethylation, could result in a lesser degree of trophoblast invasion, eventually resulting in hypoperfusion of the placenta.[119] Apart from this route, TIMP3 also has anti-angiogenic properties by blocking the binding of VEGF to VEGF receptor-2, also potentially contributing to the adverse placental conditions identified for preeclampsia.[120] Since this pregnancy condition has been associated with prenatal exposure to (BC) ambient air pollution [121,122], the link between BC air pollution exposure during gestation and TIMP3 in association with the occurrence of preeclampsia should be further investigated, since the ten mothers in our high exposure group did not experience this condition during their pregnancy.

Pathway enrichment analysis identified the ECM-receptor interaction pathway, which contains 88 protein coding genes, with COL4A2 and GP1BB as overexpressed targets in our analyses. This ECM-receptor interaction system has been identified as being overexpressed in several cancer models, such as pulmonary adenocarcinoma (especially in non-smokers) [123], breast cancer [124], and natural killer/T-cell lymphoma.[125] It is assumed that an overexpression of ECM components results in solid stress in tumors, and contributes in compression of blood vessels, reducing perfusion and consequently accessibility to therapeutics.[126,127] Moreover, in a $PM_{2.5}$ exposure-induced lung injury model in

rats, Zhang and colleagues recently found the ECM-receptor interaction pathway to be upregulated after seven days of $PM_{2.5}$ exposure.[128] Since reduced perfusion in placental tissue is a known characteristic of for example preeclampsia pregnancies [129], we would advise future research to investigate perfusion of the placenta in association with ambient BC air pollution exposure.

Hemostasis and fibrin clot formation came forward as the two enriched pathways with the lowest false discovery rate (i.e. the lowest chance of these pathways to be discovered by chance) within our enrichment analysis. The effect of prenatal air pollution exposure on placental hemostatis and fibrin clotting has not been studied to date. However, maternal smoking during pregnancy has been found to increase the creation of fibrin clots and disturb fibrin deposition in placental tissue, potentially affecting the transport of oxygen and nutrients towards the fetus.[130] The effects of air pollution on fibrin clotting have been described on the systemic hemostatic process. In a group of 137 non-smokers with both type 1 and type 2 diabetes, an IQR increase of 39.2 $\mu g/m^3$ in PM_{10}, measured 2 hours before patient examination, was associated with a 21.1 sec decrease in PFA-100 closure time, which is an indication for increased platelet activation. For long-term exposure, an area increase of 0.25 μm^2 in carbon load of airway macrophages was associated with an increase of 687 leukocytes per microliter of blood.[131] Also for long-term BC exposure, increasing BC levels were associated with an increase in fibrinogen measured in plasma samples from 6814 adults of the Multi-Ethnic Study of Atherosclerosis (MESA) study.[132]

STRING analysis of the proteins with a ≥ 1.5-fold difference in expression, shows a GO classification of the proteins into several functional groups. In the group of proteins showing a higher expression in the group of mothers prenatally exposed to high levels of BC, we observe a protein functionality which is more directed towards vascular and structural functioning of the placenta. From a vascular perspective, 5 proteins (SERPINE1, FLT1, COL4A2, ECM1, and ITGB3) were involved in angiogenesis of which 3 proteins (FLT1, ECM1 and ITGB3) have a known function in endothelial proliferation. A potential mechanism for increased expression of these proteins following BC exposure is the activation of hypoxia-related responses. It is known that exposure to air pollution can trigger hypoxic conditions in several tissues, such as the placenta, which in turn can lead to

altered development of for example lung tissue.[133] Placental hypoxia can be considered a normal event during several phases of pregnancy and is required for proper placentation and angiogenesis. However, when decreased oxygen pressure occurs in other developmental stages, this can lead to inadequate fetal cardiovascular and neurological development.[134] SERPINE1 protein levels have been shown to increase under low oxygen levels in placental tissue cell cultures, but also ITGB3, ECM1 and FLT1 expression have been shown to increase under hypoxic conditions.[135–138]

Another important group of GO classifications involved cellular interaction. Of the 41 proteins with placental overexpression in highly exposed women, 9 proteins could be linked to the negative regulation of cellular signal transduction. Seven proteins were linked to the (positive or negative) steering of cellular migration and 2 proteins, fibrillin-1 (FBN1) and integrin beta-3 (ITGB3), were associated with cell adhesion mediated by integrin. It is assumed that a disturbance of the balance between contractile structures and extracellular matrix components such as FBN1 and ITGB3 can alter proper functioning of the placenta. For example, in the placentae of preeclampsia pregnancies, there is a significant overexpression of FBN1.[139] Thirdly, 15 of the overexpressed proteins were associated with a cellular stress response, of which 6 proteins were more specifically related to the process of wound healing, namely ITGA2B, CD151, GP1BB, SERPINE2, NACA, and ITGB3.

When taking into account the proteins with an at least 2-fold change difference in expression, the proteins identified only showed overexpression in the placentas of mothers exposed to high levels of BC exposure during pregnancy. However, when we included proteins with at least 1.5-fold expression change, we found several proteins with a significantly lower placental expression. In this group of 18 proteins, STRING functional pathway analysis shows that 7 proteins were associated with the mitochondrion, namely Protein SCO1 homolog (SCO1), 3-ketoacyl-CoA thiolase (ACAA2), Glycine cleavage system H protein (GCSH), Putative transferase CAF17 (IBA57), Enoyl-CoA hydratase domain-containing protein 3 (ECHDC3), Delta-1-pyrroline-5-carboxylate dehydrogenase (ALDH4A1), and SRA stem-loop-interacting RNA-binding protein (SLIRP). The effect of air pollution exposure on mitochondria in the placenta has been studied mostly on

the level of the mitochondrial DNA (mtDNA). In the ENVIR*ON*AGE cohort, increased exposure to $PM_{2.5}$ air pollution over the entire pregnancy was associated with a 15.6% decrease in placental mtDNA content and increased mtDNA methylation, which is indicative of decreased DNA expression in the mitochondrion.[61] The same relation was found for prenatal NO_2 exposure, with a 4.9% decrease in placental mtDNA content for every 10 μg/m^3 increment in NO_2 exposure during the entire pregnancy period. This study also showed that mtDNA content was associated with birth weight in the Spanish INMA cohort, with mtDNA content as a mediatory effect in this relationship.[48]

In subsequent research, the differently expressed proteins identified in this study should be verified both on the protein level, for example by means of Western Blot, and on different expression levels, such as mRNA expression. Until recently, it was assumed that the expression of genes on the protein level was an exact mirror image of the expression on the RNA level. This view has been proven inaccurate in various organisms and tissues [140], such as the human placenta.[141] Therefore, looking solely at the proteome does not give a complete image of the association between environmental exposure and changes on the molecular level. Furthermore, future investigations should focus on the associations of other fractions of air pollution and other (daily) environmental stressors on the placental proteome to be able to complete the molecular puzzle on the effects of the environment on placental functioning and, consequently, the potential effects on human health.

This study has several strengths and limitations. We are the first to describe the relationship between prenatal exposure to ambient air pollution and the associated changes in the placental proteome. Secondly, we were able to use very accurate data on the ambient BC exposure of our participants, with the mothers being exposed to relatively low average annual ambient BC concentrations (ranging between 0.63 - 2.42 μg/m^3).[34] Measuring the BC particle load of placental samples provides a direct and exact measure of BC exposure during pregnancy, which is a great strength in this project. A limitation of this study is that, although we selected individuals with similar variables in both the high and low exposure group, there is a possibility that other variables could influence the observed

effects. Secondly, we could only examine and compare the placental proteome for 20 individuals. Taking into account technical replicates and the machine time needed for each sample, current techniques are limited in the amount of analyses that can be performed. As techniques progress, research should focus on measuring a larger number of participants in prospective birth cohorts.

In conclusion, black carbon exposure during the gestational period has a clear and profound effect on the placental proteome. Air pollution exposure affected several types of pathways, both of molecular and structural in nature. Future projects should direct their focus on whether these changes on the placental protein level have physiological implications for children's health later in life.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Proteins with an at least 1.5-fold change in expression between the high and low black carbon exposure group.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides	Group
Cellular nucleic acid-binding protein	CNBP	P62633, A0A0S2Z4K2	76.98	3	Low
Transmembrane 9 superfamily member 3	TM9S3	Q9HD45, A0A024QYS2	75.44	2	High
Plakophilin-2	PKP2	Q99959	73.59	2	High
Cystatin-C	CYTC	P01034	68.46	4	Low
SRA stem-loop-interacting RNA-binding protein	SLIRP	Q9GZT3, H0YJ40, A0A087WUN7, G3V4X6, G3V2S9	63.22	4	Low
60S ribosomal protein L6	RPL6	Q9HBB3, Q8N5Z7, Q02878, A0A024RBK3, Q8TBK5	56.35	3	High
Plasma membrane calcium-transporting ATPase 4	ATP2B4	P23634, A0A024R968	53.40	3	High
Delta-1-pyrroline-5-carboxylate dehydrogenase	ALDH4A1	P30038, A0A024RAD8	48.81	14	Low

Continued

Supplemental Table 1. Continued.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides	Group
Platelet glycoprotein Ib beta chain	GP1BB	P13224, A0A140GX60, A0A140GX63	48.79	3	High
Keratin, type I cytoskeletal 17	KRT17	Q04695	48.30	6	High
B-cell receptor-associated protein 31 variant	N/A	Q53HT6	46.75	8	High
PHD finger-like domain-containing protein 5A	PHF5A	Q7RTV0, A0A024R1U2	46.50	2	Low
Nascent polypeptide-associated complex subunit alpha	NACA	E9PAV3	46.13	4	High
Dihydropyrimidinase-like 2	N/A	Q86U75	44.34	3	Low
Keratin type I cytoskeletal 10	KRT10	P13645	43.67	21	High
Amiloride-sensitive amine oxidase [Cu-containing]	AOC1	P19801, D3DX01	43.40	18	High
CD151 antigen/Tetraspanin	CD151	P48509, A0A024RCB3	43.38	2	High
cAMP-dependent protein kinase type I-alpha regulatory subunit	PRKAR1A	P10644, B2R5T5	42.64	4	Low
Fibrinogen-like protein 1	FGL1	Q08830	41.96	12	High

Continued

Supplemental Table 1. Continued.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides	Group
ATP synthase subunit a	ATP6	H9SNR7, G4VZQ0, H9SRX2, A0A1J0PRQ1, A0A059RLI7, B2XM45, A0A141ZRP8, Q6UG00, A0A1B1PEM5, A0A059QR71, A0A343HCH7, Q4ZFC1, H9MSB7, A0A410RF61, F6N1T4	41.87	2	High
Glutaredoxin-1	GLRX	P35754, A0A024RAM2	41.51	8	Low
3-ketoacyl-CoA thiolase (mitochondrial)	ACAA2	A0A0B4J2A4, B2RB23, P42765	40.42	16	Low
Integrin alpha-IIb	ITGA2B	P08514	40.37	22	High
Bone morphogenetic protein 1/Metalloendopeptidase	BMP1	A5PLK9, P13497	38.85	2	High
Serum amyloid P-component/Pentaxin	APCS	V9HWP0, P02743	38.20	7	High
Complement factor H-related protein 1	CFHR1	Q03591	36.8	12	High
Non-POU domain-containing octamer-binding protein	NONO	A0A0S2Z4Z9, Q15233, A8K525	34.41	3	High

Continued

Supplemental Table 1. Continued.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides	Group
Solute carrier family 2 facilitated glucose transporter member 3	SLC2A3/GLUT3	P11169, B7Z5A7, B7Z966, Q59F54	34.34	3	High
Tubulin beta-1 chain	TUBB1	Q9H4B7	33.59	3	High
Putative transferase CAF17 (mitochondrial)	IBA57	Q5T440	33.54	2	Low
ADP-ribose glycohydrolase	MACROD1	Q9BQ69	33.53	2	Low
Secreted phosphoprotein 24	SPP2	Q13103	33.47	5	High
Sushi domain-containing protein 2	SUSD2	Q9UGT4, A0A140VJW3	33.41	2	Low
Extracellular matrix protein 1/Testicular tissue protein Li 61	ECM1	Q16610, A0A140VJI7	33.39	8	High
cDNA FLJ54854 highly similar to Junctional adhesion molecule A	N/A	B7Z5W1	33.05	9	High
Eukaryotic translation initiation factor 2A	EIF2A	F8WAE5, Q9BY44	32.8	2	Low
Metalloproteinase inhibitor 3	TIMP3	P35625	32.53	7	High
Fibrillin-1/ Epididymis secretory sperm binding protein	FBN1	P35555, A0A384MDM4	32.5	64	High

Continued

Supplemental Table 1. Continued.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides	Group
Keratin type II cytoskeletal 2 (epidermal)	KRT2	P35908	32.41	17	High
Glia-derived nexin; Serine protease inhibitor	SERPINE2	P07093, A0A024R451	31.12	10	High
Glycine cleavage system H protein (mitochondrial)	GCSH	A0A1W2PQV2, P23434, Q53XL7, Q6QN92	30.49	2	Low
Collagen alpha-2(IV) chain	COL4A2	P08572, A0A024RDW8	30.41	2	High
Prefoldin subunit 5	PFDN5	Q99471	30.05	2	Low
Cytidine deaminase	CDA	P32320	29.57	2	Low
Tachykinin-3	TAC3	Q9UHF0, A0A024RB47	29.32	4	High
Neuroblast differentiation-associated protein	AHNAK	Q09666	28.47	127	High
Palmitoleoyl-protein carboxylesterase NOTUM	NOTUM	Q6P988	27.65	2	High
Histone H2B type F-S	H2BFS	P57053	27.22	3	High
Plasminogen activator inhibitor 1/Serpin peptidase inhibitor clade E (Nexin plasminogen activator inhibitor type 1) member 1, isoform CRA_b	SERPINE1	P05121, A0A024QYT5	26.79	8	High

Continued

Supplemental Table 1. Continued.

Full protein name	Short name	UniProt accession(s)	Significance	Unique peptides	Group
Protein SCO1 homolog (mitochondrial)	SCO1	O75880	26.52	2	Low
Unconventional myosin-Ib	MYO1B	E9PDF6, O43795, B0I1S9	25.73	3	High
Integrin beta-3	ITGB3	P05106, L7UUZ7	25.52	17	High
Bone marrow proteoglycan	PRG2	P13727	25.29	11	High
Utrophin	UTRN	P46939	24.47	2	High
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit	STT3A	P46977, A0A024R3J7	24.42	7	High
Signal transducer and activator of transcription 1-alpha/beta	STAT1	P42224	24.28	7	High
Rabankyrin-5	ANKFY1	Q9P2R3	23.24	16	High
Fms-related tyrosine kinase 1 (Vascular endothelial growth factor/vascular	FLT1	L7RSL3, P17948	22.13	10	High
Enoyl-CoA hydratase domain-containing protein 3/ Testis tissue sperm-binding protein	ECHDC3	Q96DC8, A0A140VKF9	20.5	2	Low

The Group column indicates whether the expression of the protein is the highest in the high or in the low black carbon exposure group.

Supplemental Table 2. KEGG database results of the STRING pathway enrichment analysis for proteins with at least 1.5-fold placental overexpression in association with high prenatal black carbon exposure.

Pathway ID	Pathway description	Genes involved	False discovery rate
hsa04512	ECM-receptor interaction	COL4A2,GP1BB,ITGA2B,ITGB3	0.0016
hsa04510	Focal adhesion	COL4A2,FLT1,ITGA2B,ITGB3	0.0152
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	ITGA2B,ITGB3,PKP2	0.0152
hsa04640	Hematopoietic cell lineage	GP1BB,ITGA2B,ITGB3	0.0162
hsa04933	AGE-RAGE signaling pathway in diabetic complications	COL4A2,SERPINE1,STAT1	0.0162
hsa04611	Platelet activation	GP1BB,ITGA2B,ITGB3	0.0229
hsa05165	Human papillomavirus infection	COL4A2,ITGA2B,ITGB3,STAT1	0.0355
hsa04151	PI3K-Akt signaling pathway	COL4A2,FLT1,ITGA2B,ITGB3	0.0431

Abbreviations: GO, Gene ontology; KEGG, standard deviation.

CHAPTER 2

APPENDIX

Unidentified proteins

Within this project, it was difficult to estimate the number of unidentified proteins present in the placental tissue samples. The PEAKS software does not allow to calculate this exact number, and since there is currently no general consensus on the total number of proteins present in placental tissue, and hence no reference number to use in a calculation, the number of unidentified proteins is difficult to calculate. However, this number can be estimated in part by using the information present on the peptide level. In our experimental setting, 45% of all masses corresponding to peptides were identified with the PEAKS software. The unidentified peptide sequences could result from parameters inherent to the mass spectrometer, such as low sensitivity or poor peptide fragmentation. Furthermore, a substantial part of the inability to identify the peptides can also result from post-translational modifications or non-specific digestion by trypsin. Finally, the criteria set in the PEAKS software, such as the minimum number of peptides for protein identification or the pre-set false discovery rate (FDR), can increase or reduce the final number of proteins identified. If all the statistical thresholds would be set at the minimum, the number of identified proteins could increase by about 30%. However, this would have implications for the correctness and reliability of the data, and hence this action is avoided.

CHAPTER 3

PLACENTAL *TRPC6* EXPRESSION AND GESTATIONAL TRIMESTER-SPECIFIC $PM_{2.5}$ AIR POLLUTION EXPOSURE IN THE ENVIR*ON*AGE BIRTH COHORT

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ABSTRACT

Background: Exposure to particulate matter air pollution during pregnancy contributes to the developmental origin of various disease patterns. Alterations in placental gene expression have been linked to adverse birth outcomes and can be triggered by *in utero* air pollution exposure. TRP channels are a family of proteins involved in calcium uptake, of which TRPC6 is prevalent in human placental tissue. However, to date, the placental response of these channels to air pollution exposure during pregnancy has never been examined. We investigated the association between the placental expression of *TRPC6*, and prenatal exposure to ambient $PM_{2.5}$ air pollution (particulate matter with a diameter ≤ 2.5 µm).

Methods: We measured the relative expression of placental *TRPC6* in 159 randomly selected participants of the ENVIR*ON*AGE birth cohort by means of real-time qPCR, and we verified the results on the protein level by Western Blot. We modeled daily prenatal $PM_{2.5}$ exposure levels based on each participant's home address using a high-resolution spatiotemporal model. To identify potential critical sensitive exposure windows, we applied distributed lag models to assess the relations between placental *TRPC6* expression and weekly averaged prenatal exposure to $PM_{2.5}$.

Results: A 5 µg/m³ increment in $PM_{2.5}$ exposure during the third trimester of pregnancy was associated with a 26.6% increase [95% confidence interval (CI): 12.1% to 43.0%; p = 0.0001] in placental *TRPC6* expression at birth, with the strongest effect in gestational weeks 32 to 39. Furthermore, *TRPC6* mRNA and protein expression were inversely correlated, and TRPC6 protein expression displayed a negative association with $PM_{2.5}$ exposure between weeks 3 - 15 and weeks 27 - 35 of pregnancy.

Conclusions: Our results show that placental *TRPC6* expression exerts a trimester-specific response to prenatal $PM_{2.5}$ exposure. Further research should elucidate whether these findings could provide a new fundamental mechanism to explain the developmental origin of particle-induced disease.

INTRODUCTION

A growing body of evidence demonstrates that particle-induced effects on health and disease are rooted in a dysfunctional fetal development, which already becomes apparent in the molecular setup of the placenta. Molecular changes on the genomic, transcriptomic, proteomic, and epigenomic level have been linked to the direct or indirect effects of prenatal air pollution.[97] Ambient particles such as black carbon can be detected in human urine [106] and have the potential to translocate to all organ systems including the fetal side of the placenta.[34] This *in utero* exposure to particulate air pollution has been shown to increase oxidative stress and inflammatory markers in placenta [26] and other tissues [142,143], which potentially leads to molecular placental modifications and biomolecular ageing of the next generation.[35]

Proper placental functioning is crucial for adequate fetal development. In this context, our research group found that mitochondria in placental tissue are influenced by gestational exposure to particulate matter air pollution.[44,76] Interestingly, placental mitochondrial DNA content has been shown to predict childhood IQ, showing the clinical importance of proper energy production in placenta for long lasting clinical consequences.[144] Maternal mineral intake needs to be channeled via placental transport to ensure sufficient mineral metabolism in the growing embryo.[145] An imbalance of the placental calcium homeostasis, as shown by changes in the expression of several calcium exchange mechanisms, such as TRP channels, have been linked to detrimental pregnancy conditions such as preeclampsia.[146] TRP channels are a family of cation transport channels which are divided into six subfamilies: the 'canonical' (TRPC), 'vanilloid' (TRPV), 'melastatin' (TRPM), 'polycystin' (TRPP) 'mucolipin' (TRPM) and 'ankyrin' (TRPA) channels.[147] They are able to detect oxidative stress, the presence of chemicals and changes in temperature levels [148] and each subtype of TRP channel can be found in specific tissue types. Several members of the TRPC and TRPV subfamilies have been identified in placental tissue, while the expression of the other subfamilies is low or even nonexistent in the (human) placenta.[149]

TRPC6 is a non-selective cation channel, about six times more permeable for Ca^{2+} than for Na^{+}, and is expressed in a variety of tissues, with the highest expression

present in the lung, ovary, spleen, brain and placenta.[150] Altered expression of this channel has been linked to autism spectrum disorders [151] and a hereditary form of kidney disease.[152] The role of TRPC6 in the human placenta has not been explored to a great extent. In a *Trpc6*$^{-/-}$ mouse model depletion of this channel resulted in structural alterations in the placenta as well as in a smaller litter size compared to the control condition.[153] In human models, TRP channel expression has been investigated in association with air pollution exposure in lung tissue [154], but to our knowledge the link between placental TRP channel expression and air pollution has not yet been elucidated.

By means of the prospective ENVIRonmental influence *ON* AGEing in early life (ENVIR*ON*AGE) birth cohort, we investigated the hypothesis that prenatal exposure to $PM_{2.5}$ air pollution alters the expression of the TRPC6 channel in the placenta.

MATERIALS AND METHODS

Study population

The mother-child pairs of this study population are part of the ongoing prospective ENVIR*ON*AGE birth cohort. A detailed description on data collection is provided within the cohort profile paper by Janssen and colleagues.[88] The study protocol was approved by the ethics committees of the East-Limburg Hospital (Genk, Belgium) and Hasselt University (Diepenbeek, Belgium) and all participants signed an informed consent form before giving birth. Two hundred and forty-two mothers were recruited at the maternity ward between 12 am on Fridays and 7 am the subsequent Mondays, in the period between the 16th of April 2010 until the 12th of January 2014. The participation rate of this study was 61%. Only women with singleton pregnancies who were able to fill in a questionnaire in Dutch were eligible for participation. The midwives recorded main reasons for non-participation, including communication difficulties, labor complications, and the inability to ask for participation.

A questionnaire filled in by the participants after delivery provided us detailed information on the mothers' age, weight, height, pre-pregnancy body mass index

(BMI), education level, and her smoking habits and residential address(es) throughout the entire pregnancy. Furthermore, details on the neonate's date of birth, gestational age, sex, birth weight, and birth length were obtained from the its medical files. Maternal education, as a proxy for socio-economic status, was categorized as low (no diploma or primary school), middle (high school diploma), and high (college or university degree). Smoking status was encoded as non-smokers (both before and during pregnancy), stopped smoking before pregnancy, and mothers who smoked during pregnancy. Ethnicity was defined by the native country of the newborn's grandparents and was either coded as European (if two or more grandparents were European) or non-European (when at least three grandparents were of non-European origin).

Exposure assessment

Regional background levels of $PM_{2.5}$ (μg/m³) were determined based on the residential address(es) of the mothers during pregnancy, with the Kriging spatial-temporal interpolation method.[155] As described by Lefebvre and colleagues, this method uses air pollution data provided by 34 official fixed monitoring stations in the Flemish part of Belgium, in combination with both a dispersion model and land cover data obtained from satellite images of the CORINE land cover dataset.[104,105] This model chain provides interpolated $PM_{2.5}$ values from the Belgian telemetric air quality networks on a dense, irregular high-resolution receptor point grid from both point sources (such as industrial sites), and line sources (such as highways or major roads). Overall model performance was assessed by leave-one-out cross-validation. These validation statistics explained more than 80% of the temporal and spatial variability for $PM_{2.5}$ in Flanders, Belgium.[104,156] Moreover, the accuracy of the exposure models was recently demonstrated by the association between the urinary load of nano-sized black carbon particles in children and their residential levels of $PM_{2.5}$ and NO_2 [106] and the association between residential and placental black carbon levels.[34] The conception date was estimated by the first day of the mother's last menstrual period and ultrasound imaging data.[44] For the participants who moved during pregnancy (n = 26, 10.7%), we calculated the

exposures for both addresses, allowing for the changes in exposure during the corresponding period.

Placental tissue sampling

Placentas were collected and immediately deep-frozen by the midwives of the East-Limburg Hospital within 10 min after the birth. Tissue samples of 1 to 2 cm^3 were taken according to a standardized method at four fixed sites on the fetal side of the placenta, 4 cm from the center of the umbilical cord. All samples were taken 1.0 cm to 1.5 cm beneath the chorio-amniotic membrane and were either snap-frozen immediately and kept at -80°C until protein extraction, or transferred into RNAlater (Qiagen, KJ Venlo, the Netherlands) and kept at –20°C until RNA extraction, after an incubation at 4°C for 24 hours.

RNA extraction, cDNA synthesis and Real-time PCR

We pooled samples from the placental biopsies (± 25 mg final weight) taken from all four fixed sites to reduce potential intra-placental differences in TRP channel expression, since placental gene expression can vary between sampling sites.[157] Total RNA was extracted with the miRNeasy mini kit (Qiagen, KJ Venlo, the Netherlands) according to the manufacturer's instructions. RNA quantity and purity of the samples was measured by means of spectrophotometry (Nanodrop ND-1000; Isogen Life Science, De Meern, the Netherlands). To minimize potential DNA contamination, we performed a DNase treatment on the RNA extracts according to the manufacturer's protocol (Turbo DNA-free kit, Ambion, Life Technologies, Diegem, Belgium). Treated RNA samples were stored at -80°C until cDNA synthesis.

Extracted RNA, with an input of 3 µg, was reverse transcribed to cDNA with the GoScript Reverse Transcription System (Promega, Madison, WI, USA) using a Veriti 96-well Thermal cycler (TC-5000; Techne, Burlington, NJ, USA), according to the manufacturer's instructions. cDNA samples were stored at –20°C until further applications. Quantitative real-time PCR analyses were performed for

TRPC6 as target gene and *GAPDH*, *IPO8*, *UBC*, and *POLR2A* as reference genes (**Supplemental table 1**). All samples were measured in triplicate. In short, 2 µl of cDNA sample (3 ng/µl) was mixed with 5 µl of TaqMan Fast Advanced Master Mix (Life Technologies, Diegem, Belgium) and 0.5 µl of primer assay (Integrated DNA Technologies, Coralville, Iowa, USA) in a final reaction volume of 10 µl. Subsequently, the samples were amplified with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), according to a standard thermal cycling scheme of 1 cycle of 2 min at 50°C for AmpErase® UNG activation, 1 cycle for 10 min at 95°C to activate the AmpliTaq Gold® DNA Polymerase enzyme, 40 cycles of 20 sec at 95°C for denaturation and 1 min at 60°C for annealing of the primers and sequence extension.

Obtained Ct (cycle threshold) values of *TRPC6* were entered in the qBase software (Biogazelle, Zwijnaarde, Belgium) for normalization, relative to the four measured reference genes. This software uses the comparative Ct method (ΔΔCt) method while equally taking into account the expression of inter-run calibration algorithms to correct for run-to-run plate differences.[158] Technical replicates were excluded when the difference in Ct value was greater than 0.5. When this technical variation was > 0.5, samples were measured a second time. Samples that not met quality control criteria of the qBase software, i.e. with a technical variation > 0.5 or Ct values > 35, for either the target genes or (one of) the genes used for normalization, were excluded from the dataset. After running the qBase analyses on the remaining participants, of the original set of 242, we obtained a normalized measurement for *TRPC6* in 159 individuals.

Protein extraction and Western Blot

Proteins were extracted from a pooled tissue sample of 40 mg in 300 µL extraction buffer, consisting of 2% CHAPS, 2% DTT, 2 M thiourea and 7 M urea. Tissue samples were disrupted mechanically with an Ultra Turrax T8 mixer (IKA, Staufen, Germany) three times during 10 sec. Subsequently, the lysates were incubated on a shaking plate for 30 min at 1400 rpm and 15°C and then centrifuged for 5 min at 16.000 g. Protein concentration was determined in the resulting supernatant with Ionic Detergent Compatibility Reagent (Thermo Scientific,

Massachusetts, USA) dissolved in Pierce™ 660 nm Protein Assay Reagent (Thermo Scientific). Protein extracts were kept at -80°C until used for Western Blot.

For the Western Blot, we used mouse brain extracts as positive control and the Color Prestained Protein Standard, Broad Range (New England BioLabs, Massachusetts, USA) as protein standard. Twenty micrograms of each extract was loaded onto a 4% - 20% Mini-PROTEAN® TGX™ Precast Protein gel (10 well - 30 µl, ref #4561093, Bio-Rad, California, USA), which were run for 40 min at 200 V. Subsequently, proteins were blotted with the Trans-Blot® Turbo™ RTA Mini LF PVDF Transfer Kit (Bio-Rad), according to the manufacturers' instructions. We used the fluorescent immunostaining of actin as loading control. Primary antibodies used for this experiment were rabbit anti-TRPC6 (#16716, Cell Signaling Technology, Massachusetts, USA) and anti-β-actin (#A5441, Sigma-Aldrich, Missouri, USA), and IRDye 800CW-conjugated goat anti-rabbit (#926-32211, LI-COR Biotechnology, Nebraska, USA) was used as secondary antibody. Both primary antibodies were diluted in Odyssey Blocking Buffer (PBS) (LI-COR Biotechnology) and 0.1% Tween (1000x for TRPC6 and 20,000x for anti-β-actin) and incubated onto the membrane, overnight at 4°C for TRPC6 and 30 min at room temperature (RT) for anti-β-actin. The secondary antibody was diluted 10,000 times in the same medium, and incubated at RT for one hour. Imaging and relative quantitative fluorescence analyses were performed with the Odyssey Classic Infrared Imaging System (LI-COR Biotechnology) (**Supplemental Figure 1**). Normalized TRPC6 expression values were obtained by calculating the ratio of gene expression values: $\frac{placental\ TRPC6/actin}{placental\ TRPC6/mouse\ brain\ TRPC6}$

Statistics

Statistical analysis was performed in SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA) and in R (version 3.6.1) using the "dlnm" package in the latter.[159] Unadjusted associations between $PM_{2.5}$ exposure and TRP channel expression, and *TRPC6* mRNA and protein expression were measured with Pearson correlation coefficients. In these analyses, we looked at the daily $PM_{2.5}$ levels averaged over each of the three trimesters of pregnancy. To test adjusted associations between prenatal $PM_{2.5}$ exposure and TRP channel expression we used distributed lag models (DLMs) [160] in which we modelled simultaneously the level of $PM_{2.5}$ exposure during gestation and the lagged association with TRP expression. We specified for the lag space a natural cubic spline with 3 equally spaced inner knots between week 1 and week 40 of pregnancy and for the exposure space a linear relationship. All models were adjusted for newborn's sex, gestational age (weeks), birth weight, ethnicity, month of birth, maternal age (years), pre-pregnancy BMI, maternal smoking behavior during pregnancy and education level as an indicator for socio-economic status. The magnitude of all associations was expressed for a 5 $\mu g/m^3$ increment in prenatal $PM_{2.5}$ exposure.

RESULTS

Demographic and exposure characteristics of the study population

Demographic specifications of the 159 mother-newborn pairs included in this study are described in **Table 1**. Average (SD) maternal age at the moment of child birth was 29.1 (4.2) years old. Most of the mothers (64.2%) were non-smokers and highly educated (54.7%). The neonates (53.5% females) had an average birth weight of 3413.8 (454.9) g and an average length of 50.3 (2.0) cm.

Table 1. Characteristics of the mother-child pairs included in this study (n = 159).

Characteristic	Mean (SD) or number of participants (%)
Mother	
Age at birth child (years)	29.1 (4.2)
Pre-pregnancy BMI (kg/m²)	24.4 (4.6)
Smoking behavior during pregnancy	
Never smoked	102 (64.2)
Stopped smoking before pregnancy	28 (17.6)
Smoked during pregnancy	29 (18.2)
Education level	
Low (no high school diploma)	22 (13.8)
Middle (high school diploma)	50 (31.5)
High (college degree or higher)	87 (54.7)
Neonate	
Gestational age (weeks)	39.2 (1.3)
Sex	
Male	74 (46.5)
Female	85 (53.5)
Ethnicity	
European	144 (90.6)
Non-European	15 (9.4)
Birth length (cm)	50.3 (2.0)
Birth weight (g)	3413.8 (454.9)
Season of birth	
Spring	46 (28.9)
Summer	33 (20.8)
Autumn	39 (24.5)
Winter	41 (25.8)

Abbreviations: BMI, Body mass index; SD, standard deviation.

Average $PM_{2.5}$ exposure during the three trimesters and the entire pregnancy period was similar, with a mean [SD] exposure of 13.7 [5.2] μg/m³ for the first trimester, 14.5 [4.9] μg/m³ for the second trimester, 14.8 [6.1] μg/m³ for the third trimester and 14.3 [2.3] μg/m³ for the entire pregnancy. The weekly average $PM_{2.5}$ exposure for our study population is summarized in **Table 2**.

Placental expression of *TRPC6* in association with $PM_{2.5}$ exposure

Unadjusted analyses between relative *TRPC6* expression and prenatal $PM_{2.5}$ exposure showed a significant correlation for the exposure in the first trimester ($r = -0.16$, $p = 0.04$) and the third trimester of pregnancy ($r = 0.31$, $p = < 0.0001$). From the DLM model, no significant association could be determined between $PM_{2.5}$ exposures and *TRPC6* expression during the first and second trimester. However, a 5 μg/m³ increment in $PM_{2.5}$ during the third trimester of pregnancy was significantly associated with an 26.6% increase [95% confidence interval (CI): 12.1% to 43.0%; $p = 0.0001$] in placental *TRPC6* expression at birth. A critical window of $PM_{2.5}$ exposure could be determined between the 32nd and 40th week of pregnancy (**Figure 1**).

We additionally performed a sensitivity analysis excluding mothers who actively smoked during pregnancy. For *TRPC6*, the association with third trimester $PM_{2.5}$ exposure decreased, with an 11.7% [95% CI: -0.67% to 25.6%; $p = 0.06$] increase in relative *TRPC6* expression in association with a 5 μg/m³ increment in third trimester $PM_{2.5}$ exposure, with a smaller critical exposure window between gestational weeks 33 and 38 (**Supplemental Figure 2A**).

Table 2. **Exposure details on $PM_{2.5}$ air pollution exposure (μg/m³) during different time windows of pregnancy.**

Week of pregnancy	Number of Participants	Mean (SD)	Lower limit 95% CI	Upper limit 95% CI
1	159	14.4 (10.0)	12.9	16.0
2	159	14.3 (8.7)	12.9	15.6
3	159	13.2 (8.4)	11.9	14.5
4	159	14.5 (9.7)	12.9	16.0
5	159	14.6 (10.0)	13.0	16.1
6	159	14.3 (9.6)	12.8	15.8
7	159	13.6 (8.8)	12.2	15.0
8	159	14.4 (8.5)	13.1	15.8
9	159	14.6 (8.4)	13.3	15.9
10	159	14.5 (9.3)	13.0	15.9
11	159	14.1 (8.5)	12.7	15.4
12	159	15.4 (9.8)	13.9	16.9
13	159	14.5 (8.9)	13.1	15.9

Continued

Table 2. **Continued**

Week of pregnancy	Number of Participants	Mean (SD)	Lower limit 95% CI	Upper limit 95% CI
14	159	15.5 (10.0)	13.9	17.1
15	159	15.1 (10.1)	13.6	16.7
16	159	13.8 (8.7)	12.4	15.1
17	159	14.5 (8.6)	13.2	15.9
18	159	14.6 (9.3)	13.2	16.1
19	159	15.0 (9.3)	13.5	16.4
20	159	14.7 (8.7)	13.3	16.1
21	159	15.3 (9.7)	13.8	16.8
22	159	15.4 (9.3)	14.0	16.9
23	159	15.0 (8.9)	13.6	16.4
24	159	16.2 (10.1)	14.6	17.8
25	159	15.5 (9.6)	14.0	17.0
26	159	15.2 (9.5)	13.7	16.7

Continued

Table 2. **Continued**

Week of pregnancy	Number of Participants	Mean (SD)	Lower limit 95% CI	Upper limit 95% CI
27	159	16.1 (11.3)	14.3	17.9
28	159	16.2 (10.6)	14.5	17.9
29	159	15.1 (9.1)	13.7	16.5
30	159	15.8 (10.1)	14.2	17.4
31	159	15.0 (9.2)	13.6	16.4
32	159	15.7 (10.0)	14.1	17.2
33	159	16.6 (10.1)	15.0	18.1
34	159	16.2 (10.8)	14.5	17.8
35	156	14.9 (9.4)	13.4	16.4
36	152	15.0 (10.9)	13.3	16.8
37	143	14.0 (10.3)	12.3	15.7
38	123	12.6 (9.5)	10.9	14.3
39	69	12.3 (8.5)	10.3	14.3
40	19	12.1 (6.4)	9.0	15.2

Abbreviations: CI, Confidence interval; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; SD, standard deviation.

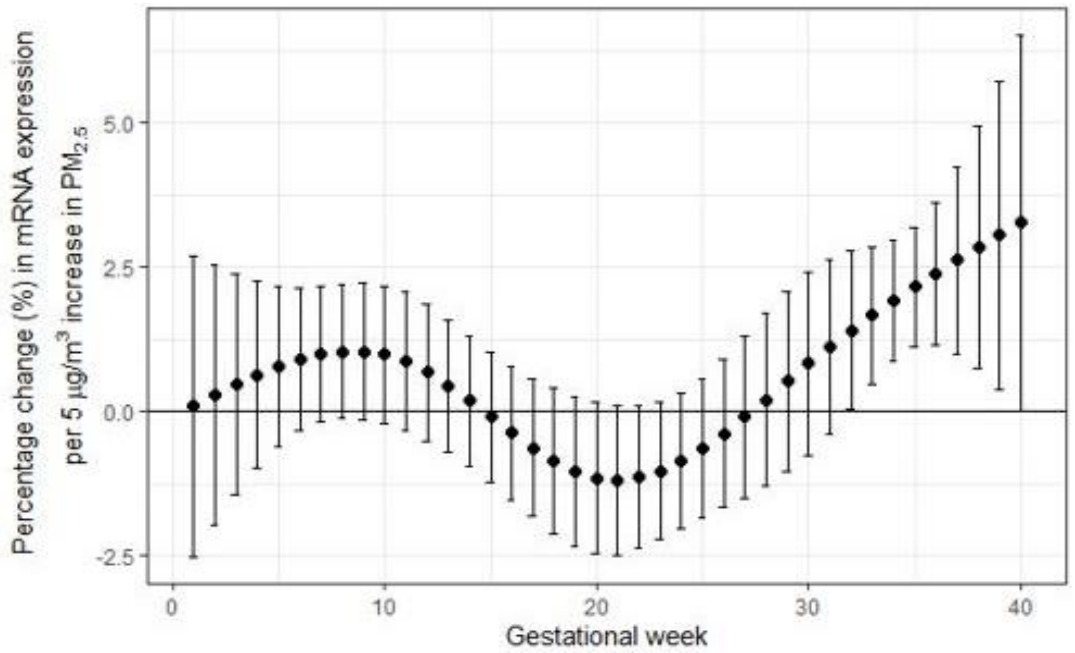


Figure 1. Relative TRPC6 expression in association with week-specific prenatal $PM_{2.5}$ exposure. Week-specific estimates are provided as a percentage change in mean relative *TRPC6* expression (log10) for every 5 µg/m^3 increase in $PM_{2.5}$ air pollution exposure. Distributed lag models were adjusted for maternal age (years) at the birth of their child, newborn's sex, gestational age (weeks), birth weight, pre-pregnancy BMI, ethnicity, month of birth, maternal smoking behavior during pregnancy and maternal education level. Abbreviations: $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; TRPC6, Transient receptor potential canonical channel 6. exposure.

Relation between relative TRPC6 expression on mRNA and protein level

As additional analysis, we examined whether the relative expression of *TRPC6* on the mRNA level could be compared with its expression on the protein level, and whether TRPC6 protein expression was associated with gestational $PM_{2.5}$ exposure as well. We obtained relative expression values on both the mRNA and protein level for 117 subjects. We observed a negative correlation ($r = -0.40$; $p < 0.0001$) between TRPC6 expression on both levels (**Figure 2A**). In association with prenatal $PM_{2.5}$ exposure, we found a significant negative correlation between third trimester $PM_{2.5}$ expression and relative protein expression levels ($r = -0.22$; $p = 0.02$). In adjusted distributed lag models, a negative association was identified between relative TRPC6 protein expression and $PM_{2.5}$ expression in the first and third trimester: -25.8% [95% CI: -35.5% to -15.0%; $p < 0.0001$] for every 5 μg/m³ increase in $PM_{2.5}$ with a critical time window between gestational weeks 4 and 15, and -11.7% [95% CI: -21.6% to -0.6%; $p = 0.04$] for every 5 μg/m³ increase in $PM_{2.5}$ with a critical time window between gestational weeks 27 and 35, respectively (**Figure 2B**).

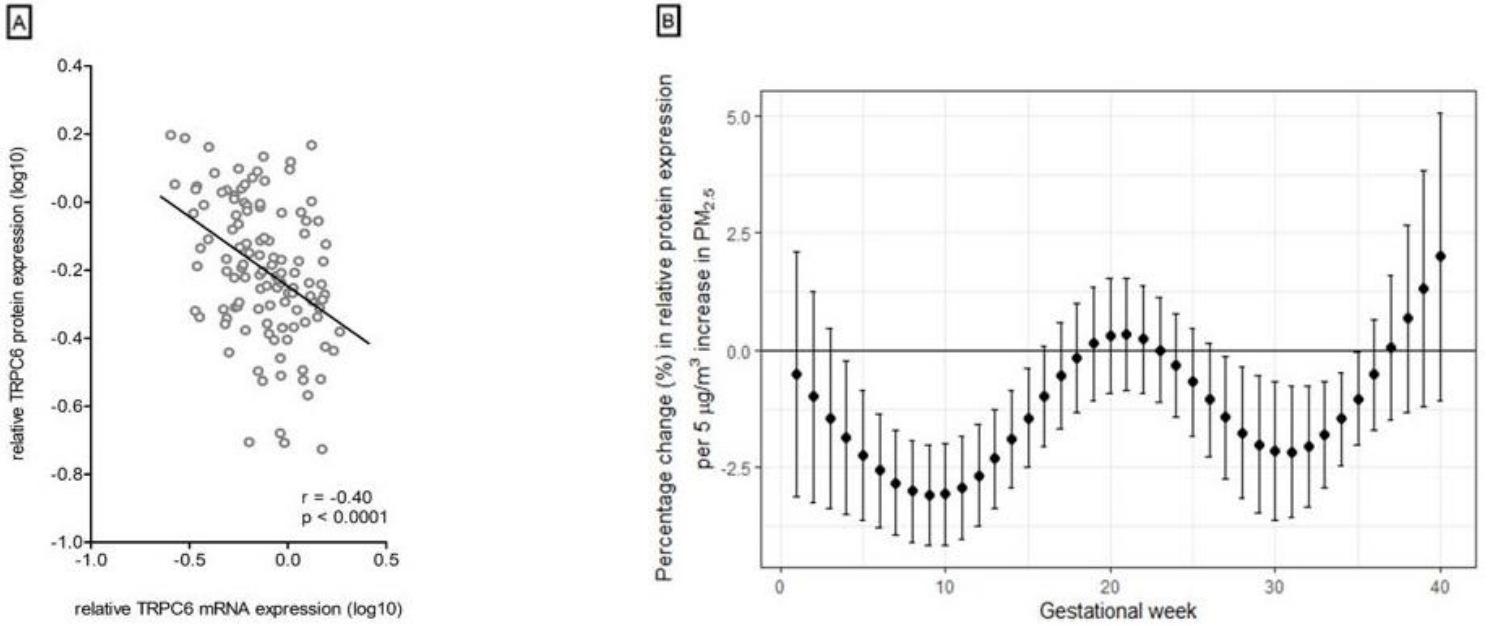


Figure 2. Relative TRPC6 protein expression in association with week-specific prenatal $PM_{2.5}$ exposure. The correlation plot shows the negative correlation between relative *TRPC6* mRNA expression and relative TRPC protein expression (Figure A). In the adjusted model, week-specific estimates are provided as a percentage change in mean relative TRPC6 protein expression (log10) for every 5 µg/m^3 increase in $PM_{2.5}$ air pollution exposure (Figure B). Distributed lag models were adjusted for maternal age (years) at the birth of their child, newborn's sex, gestational age (weeks), birth weight, pre-pregnancy BMI, ethnicity, month of birth, maternal smoking behavior during pregnancy and maternal education level. Abbreviations: $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; TRPC6, Transient receptor potential canonical channel 6.

DISCUSSION

We investigated whether the expression of *TRPC6* in human placental tissue is associated with $PM_{2.5}$ air pollution exposure during pregnancy. Our results show that there is a significant trimester-specific association between both relative *TRPC6* mRNA and TRPC6 protein expression, and $PM_{2.5}$ exposure during the third trimester of pregnancy. TRPC6 protein expression was inversely associated with *TRPC6* mRNA expression in our dataset, which was confirmed by the observation of a negative association between $PM_{2.5}$ exposure during gestational weeks 27 and 35 and TRPC6 protein expression.

To our knowledge this is the first time that human placental TRP channel expression is linked to prenatal air pollution exposure. With regard to air pollution, *TRPC6* expression has so far only been examined in lung tissue. Although especially TRPA1 and TRPV1 appear to be important chemical sensors in the respiratory system [161], a recent study on the effects of ozone exposure in a Trpc6-deficient mouse model indicated that this channel is involved in the inflammatory response in lung tissue that is generated by this component of ambient air pollution.[162] *TRPC6* expression in lungs has also been studied in association with other adverse environmental exposures such as cigarette smoke. Increased expression of this cation channel on both the mRNA and protein level was detected in rat lung tissue in association with chronic exposure to cigarette smoke, eventually leading to remodeling of the pulmonary arteries.[163] Furthermore, in an *in vitro* model of chronic obstructive pulmonary disease (COPD), adding nicotine to human bronchial smooth muscle cells doubled the expression of *TRPC6*, resulting in higher cell proliferation.[164] Although inverse for mRNA and protein expression, the association between *TRPC6* expression and $PM_{2.5}$ exposure was especially significant for the third trimester of pregnancy. Trimester-specific effects of ambient air pollution exposure have not been described for ion channel expression in the placenta, although third trimester-specific changes have been observed in for example placental phosphatase and tensin homolog (PTEN) expression [63] and the methylation pattern of several genes of the circadian pathway such as neuronal PAS domain-containing protein 2 (NPAS2), cryptochrome circadian clock 1 (CRY1), and period circadian clock 2 (PER2) and 3 (PER3).[165]

For *TRPC6*, we assessed the association with prenatal $PM_{2.5}$ exposure on both the mRNA level and the protein level. Seemingly remarkable, since mRNA sequences are translated into protein, we observed an inverse relation between *TRPC6* mRNA and protein expression levels and a significant but inverse association between each of the expression levels and prenatal $PM_{2.5}$ exposure. However, such a discrepancy between both expression levels is not uncommon. Comparing RNA and protein levels between 23 human cell lines in the research of Gry and colleagues revealed that a significant positive correlation between mRNA and protein expression only existed for a third of all screened targets.[140] Additional research on mechanisms inhibiting *TRPC6* RNA translation, such as specific microRNA expression patterns, could lead to more insight into this complex relation between *TRPC6* mRNA and protein levels, and its relation with ambient air pollution exposure.

TRP channels are sensors of oxidative stress and react to increased levels of reactive oxygen species (ROS) [148,166], resulting in an elevated Ca^{2+} influx. TRPC6 activation by ROS has been described in a recent study combining an *in vitro* human cell line and an *in vivo* rat model. ROS induced the upregulation of *TRPC6* mRNA levels and increased TRPC6-mediated Ca^{2+} influx in human and rat podocytes.[29] Particulate matter air pollution has been shown to increase the levels of ROS in several human matrices, such as blood and urine.[167] Moreover, prenatal exposure to air pollution has been linked to increased levels of 3-nitrotyrosine (3-NTp), a biomarker of oxidative stress, in human placental tissue.[26] Therefore, an increase in oxidative stress levels could be a potential mode of activation of TRPC6 as a result to *in utero* air pollution exposure.

Ca^{2+} transport, mediated by cation transporters and channels, is an important mediator in several cellular signaling pathways and Ca^{2+} plays an important role as second messenger in neurological transmission, apoptosis and the secretion of enzymes and hormones.[146] Animal models have shown that proper Ca^{2+} homeostasis is crucial already at the implantation of the blastocyst and the early development of the trophoblast.[168] Bolnick and colleagues have shown that apoptosis is triggered in a human cytotrophoblast cell line when intracellular Ca^{2+} levels are increased by ethanol exposure.[169] Research in a mouse podocyte cell line indicated that albumine triggered the activation of TRPC6, resulting in an

increased Ca^{2+} influx and a subsequent increase in podocyte apoptosis.[170] The link between $PM_{2.5}$ air pollution and apoptosis has been examined in several cell line models of the human lung [171,172] and in an *in vivo* mouse model of the cerebral effects of $PM_{2.5}$ air pollution exposure during pregnancy in the offspring.[173] Therefore, the potential role for placental TRPC6 activation as mediator between $PM_{2.5}$ exposure and placental cell apoptosis and functioning should be studied in future research.

This study has several strengths and limitations. A first strength is that these results were obtained through extensive data collection at birth and elaborate bio-banking within a large prospective birth cohort which enables us to contribute to the knowledge on the developmental origins of health and disease. Secondly, we used data from a population-based sample size of mother-child pairs, representative for the reproductive segment of the Flemish population of Belgium.[88] Thirdly, we measured both mRNA and protein expression, providing a more in depth view of the relation between environmental exposure and its cellular effects. A first limitation of this study is that, although we performed a thorough a priori selection of potential confounding factors, residual confounding can never be fully excluded. Secondly, in our analyses we use daily levels of residential particulate exposure during pregnancy, but we did not account for exposures other than residential, which could result in a potential misclassification of exposure, although this would rather minimize than enlarge our observations, as we assume misclassification to be a random process. However, the accuracy and relevance of our models for (inter)personal exposure has been proven since residential air pollution levels and proxies thereof, such as the proximity of the home to major roads, have been shown to correlate with the levels of nano-sized black carbon levels in the urine of children from the same study area.[106]

CONCLUSION

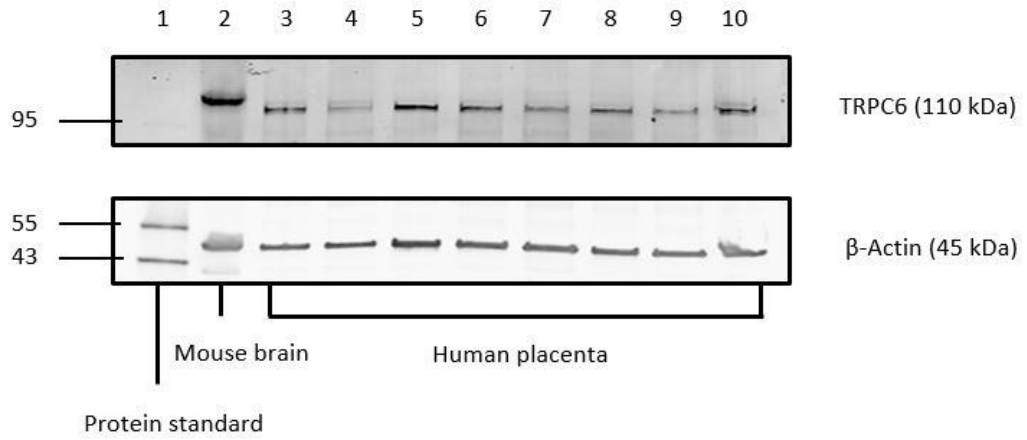
The placental TRPC6 channel is involved in the trimester-specific responses of prenatal exposure to $PM_{2.5}$ air pollution. These results could provide new insights on the complex intricate effect of ambient air pollution exposure on human health. Further research on the downstream placental effects of gestational air pollution exposure on TRPC6 function and the potential link of our results with health effects in the children born from these pregnancies could open a new window of knowledge on the developmental origin of particle-induced disease.

SUPPLEMENTAL MATERIAL

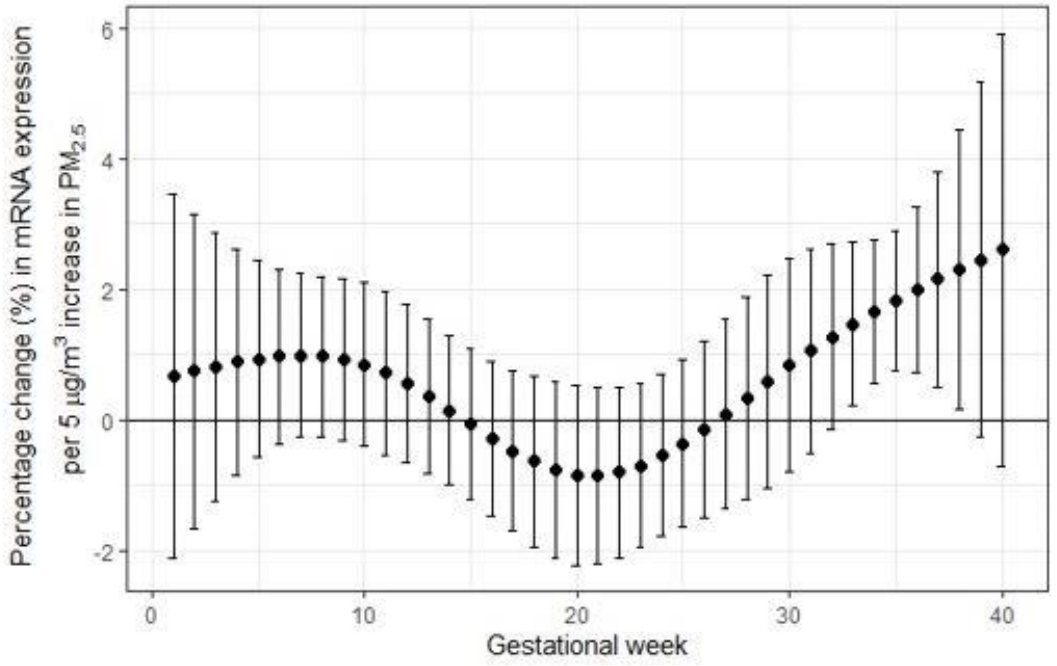
Supplemental Table 1. Assays for qPCR analyses of the used target and reference genes.

Gene	Assay Name	RefSeq Number	Exon location	Forward primer sequence	Reverse primer sequence
TRPC6	Hs.PT.58.23046270	NM_004621	7-8	5'CCCAGAACAGT GTCTTAAAACTC-3'	5'-CATTATGGTG TTTGTGGCCTT-3'
GAPDH	Hs.PT.53a.24391631.gs	NM_001256799	7-8	5'-TGAGTCCTTC CACGATACCA-3'	5'-ACCATGAGAAG TATGACAACAGC-3'
IPO8	Hs.PT.56a.40532361	NM_001190995	7-8	5'-CCACTTCTTAC ACTTCCACCAT-3'	5'-GAGATCTTCCGA ACTATTATCGACA-3'
POLR2A	Hs.PT.56a.25515089	NM_000937	23-24	5'-TCAGCATGTT GGACTCGATG-3'	5'-CGTATTCGCAT CATGAACAGC-3'
UBC	Hs.PT.39a.22214853	NM_021009	1-2	5'-CCTTATCTTGGA TCTTTGCCTTG-3'	5'-GATTTGGGTC GCAGTTCTTG-3'

TRPC6 was selected as target gene, and GAPDH, IPO8, POLR2A and UBC were selected as reference genes. For each gene, the assay, RefSeq number, exon location and the sequence of the forward and reverse primer are given.



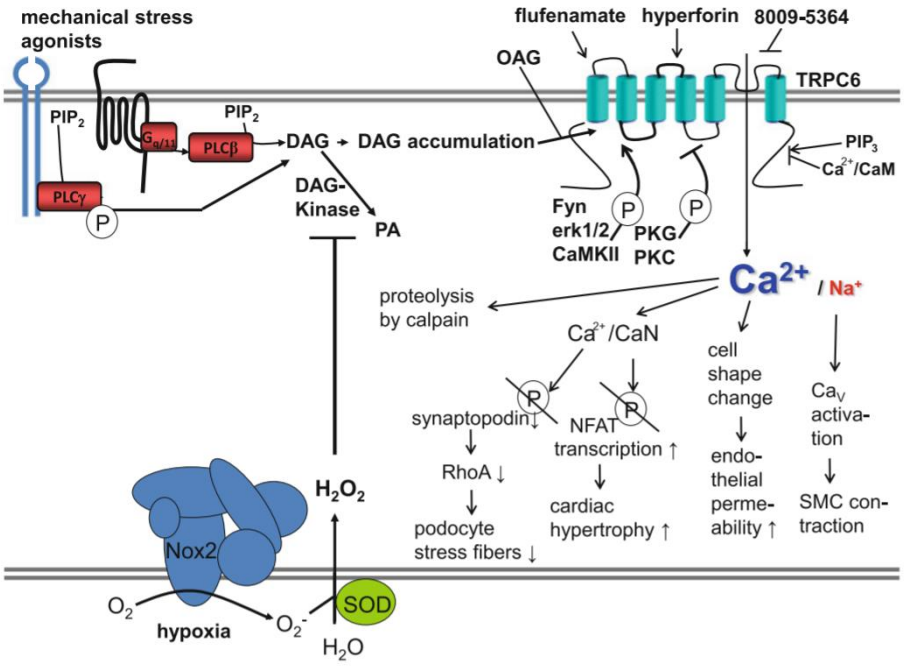
<u>Supplemental Figure 1.</u> Western Blot analysis of TRPC6 and β-Actin. Lane 1 contains the protein standard (Color Prestained Protein Standard, Broad Range). Lane 2 shows the results in mouse brain tissue and lanes 3 to 10 show the results in human placental tissue. Abbreviations: TRPC6, Transient receptor potential canonical channel 6.



<u>Supplemental Figure 2.</u> Sensitivity analysis excluding women who smoked during pregnancy (n = 29). Week-specific estimates are provided as a percentage change in mean relative TRPC6 expression (log10) for every 5 µg/m^3 increase in $PM_{2.5}$ air pollution exposure. Distributed lag models were adjusted for maternal age (years) at the birth of their child, newborn's sex, gestational age (weeks), birth weight, pre-pregnancy BMI, ethnicity, month of birth, maternal smoking behavior during pregnancy and maternal education level. Abbreviations: $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; TRPC6, Transient receptor potential canonical channel 6.

APPENDIX

The activation of TRPC6 has been verified in placental tissue, more specifically in cytotrophoblast and syncytiotrophoblast cell populations. In the latter, the activation of the channel was triggered by thapsigargin, increasing store-operated Ca^{2+} entry into the cell, followed by superfusion with a Ca^{2+}-containing solution, which strongly increased the Ca^{2+} flux especially in term placental tissue.[174] According to the summarized information by Dietrich and Gudermann, it can be concluded that TRPC6 channels can be activated both on the extracellular side and on the intracellular side of the plasma membrane (Appendix Figure 1).[150]



<u>Appendix Figure 1.</u> The transmembrane TRPC6 channel can be activated via both intracellular and extracellular factors. Activation by the accumulation of intracellular DAG levels is the most described method of TRPC6 activation, but external substances and mechanical stress can induce the activation of the cation channel as well. The resulting Ca^{2+} influx can result in several consequences for the cell, such as smooth muscle cell contraction and increased endothelial cell permeability. *Reprinted from Dietrich and Gudermann; Mammalian transient receptor potential (TRP) cation channels.*

TRPC6 is a receptor-operated channel, which can be activated by IP3 and DAG production in the PLC pathway (and, in an experimental setting, by the DAG homologue 1-oleoyl-2-acetyl-sn-glycerol (OAG)). Other substances used to activate TRPC6 for *in vitro* experiments include hyperforin, 2,4-diacylphloroglucinol and PPZ1,2.[175] It thus seems that TRPC6 channels can be activated by intracellular store-operated Ca^{2+} entry, however TRPC6 is not generally assumed to be a store-operated channel. Increased levels of diacylglycerol (DAG), triggered by an activation of the phospholipase C receptor (PLC), can also result in the activation of TRPC6 channels without any changes in internal Ca^{2+} stores.[150] Phosphorylation on the other hand, by for example protein kinase C (PKC), on specific locations of the amino acid sequence such as serine 768 has been shown to inhibit the activation of TRPC6. Some studies also suggest that TRPC6 can be activated by mechanical stress, but this is still a subject of dispute.[150,176]

Increased Ca^{2+} entry by TRPC6 channel activation has been linked to various physiological consequences for the cell. Firstly, TRPC6-triggered Ca^{2+} entry in smooth muscle cells have been shown to stimulate proliferation.[177] Indeed, an increment in intracellular Ca^{2+} seems to be an essential stimulus for proliferation to occur. More specifically, knockout and inhibition of TRPC6 in an experimental setting have indicated that TRPC6-induced calcium influx is most important for successful transition of the cell cycle in G2/M phase.[178] Secondly, especially in podocytes, several studies have indicated that TRPC6 activation and subsequent Ca^{2+} influx is involved in apoptosis. Although the exact mechanism for this still remains unclear, several mechanisms have been suggested to regulate TRPC6-triggered apoptosis, such as activation of the mTORC2/Akt/NFkB signalling pathway or increased H_2O_2 production by tipping the redox balance towards oxidative stress.[179,180]

PART 2: MICROVASCULAR CHANGES ASSOCIATED WITH (PRENATAL) AIR POLLUTION EXPOSURE

CHAPTER 4

CHILDREN'S MICROVASCULAR TRAITS AND AIR POLLUTION EXPOSURE DURING PREGNANCY AND EARLY CHILDHOOD: PROSPECTIVE EVIDENCE TO ELUCIDATE THE DEVELOPMENTAL ORIGIN OF PARTICLE-INDUCED DISEASE

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ABSTRACT

Background: Particulate matter exposure during *in utero* life may entail adverse health outcomes later in life. The microvasculature undergoes extensive, organ-specific prenatal maturation. A growing body of evidence shows that cardiovascular disease in adulthood is rooted in a dysfunctional fetal and perinatal development, in particular that of the microcirculation. We investigate whether prenatal or postnatal exposure to $PM_{2.5}$ (particulate matter with a diameter ≤ 2.5 µm) or NO_2 is related to microvascular traits in children between the age of four and six.

Methods: We measured the retinal microvascular diameters, the central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE), and the vessel curvature by means of the tortuosity index (TI) in young children (mean [SD] age 4.6 [0.4] years), followed longitudinally within the ENVIR*ON*AGE birth cohort. We modeled daily prenatal and postnatal $PM_{2.5}$ and NO_2 exposure levels for each participant's home address using a high-resolution spatiotemporal model.

Results: An interquartile range (IQR) increase in $PM_{2.5}$ exposure during the entire pregnancy was associated with a 3.85-µm (95% CI, 0.10 to 7.60; p = 0.04) widening of the CRVE and a 2.87-µm (95% CI, 0.12 to 5.62; p = 0.04) widening of the CRAE. For prenatal NO_2 exposure, an IQR increase was found to widen the CRVE with 4.03 µm (95% CI, 0.44 to 7.63; p = 0.03) and the CRAE with 2.92 µm (95% CI, 0.29 to 5.56; p = 0.03). Furthermore, a higher TI score was associated with higher prenatal NO_2 exposure. We observed a postnatal effect of short-term $PM_{2.5}$ exposure on the CRAE and a childhood NO_2 exposure effect on both the CRVE and CRAE.

Conclusions: Our results link prenatal and postnatal air pollution exposure with changes in a child's microvascular traits as a fundamental novel mechanism to explain the developmental origin of cardiovascular disease.

INTRODUCTION

A growing body of evidence shows that particulate-induced health effects are rooted in dysfunctional fetal and perinatal development.[97] At least four lines of evidence contribute to this statement. First, during the earliest phases of life, the fetus can be exposed to particulate matter via the placenta. An ex vivo human placental perfusion model has shown that trans-placental transport is able to channel particles with a diameter smaller than 240 nm.[10] In addition, an *in vivo* rabbit model exposed to diesel exhaust demonstrated that particles can even be transferred into the fetal bloodstream.[181] Accordingly, in human context, it was recently found that even low concentrations of ambient particles, including black carbon, are present in detectable quantities at the fetal side of the placenta.[34] Second, *in utero* exposure to particulate air pollution can increase oxidative stress and inflammatory markers, which potentially leads to molecular modifications in for example placental tissue.[26] Third, both DNA damage, measured by for example DNA adducts [182], elevated levels of micronuclei [183], and DNA methylation levels [99], and epigenetic markers such as DNA methylation [143], histone modification, and miRNAs expression [63], which ultimately regulate chromatin structure or gene activity, have been associated with *in utero* exposure to particulate air pollution. Finally, several postnatal effects related to prenatal particulate exposure have been elaborated in recent research, for example on molecular longevity, as reflected by telomere length [35], or on clinical factors such as birth weight.[184] In turn, these effects have been linked to (markers of) disease development later in life, such as increased stiffness of the carotid artery or an increased risk of cardiovascular disease development.[185,186]

The microcirculation makes up a large part of the human vasculature and undergoes extensive, organ-specific perinatal maturation.[187,188] Over the past years, fundus photography has increasingly been used to assess microvascular health.[189] A strong correlation exists between macrovascular and microvascular parameters in adults, which has been shown for example by Seidelmann and colleagues: in 2617 persons with the highest quartile of retinal venular diameter, contrasting the persons with the lowest venular diameter quartile, this resulted in a 2.4% higher risk of atherosclerotic events during a 10-year follow-up.[190] Therefore, changes in the microvasculature of the retina at an early age can be

an indicator of cardiovascular disease later in life.[79,80] Furthermore, particulate matter air pollution is an important risk factor for adverse cardiovascular effects later in life.[191–194] Recently, our research group described an association between short-term ambient air pollution exposure at school and an increase in retinal arteriolar diameter in 10-year-old children.[82] However, to date, no studies have considered changes in the maturation of the microvasculature in relation to prenatal exposure to particulate air pollution. By making use of the prospective ENVIRonmental influence *ON* AGEing in early life (ENVIR*ON*AGE) birth cohort, we tested the hypothesis in 4-to-6-year-olds that prenatal and postnatal particulate air pollution exposures are associated with changes in the diameter and curvature of retinal blood vessels in early childhood.

MATERIALS AND METHODS

Study population

The participants of this study are enrolled in the ongoing prospective ENVIR*ON*AGE birth cohort. Detailed information on data collection within this cohort is described in the article of Janssen and colleagues.[88] Before delivery, an initial informed consent form was signed by the mothers, and after delivery, the participants filled out a questionnaire which provided us detailed medical and lifestyle data, including their residential address(es) during pregnancy, maternal age, maternal weight and height, maternal pre-pregnancy body mass index (BMI), maternal education, and maternal smoking habits and alcohol consumption throughout pregnancy. Additionally, perinatal parameters, such as the date of birth, gestational age, sex, birth weight, and birth length, were obtained from the participant's medical files. Maternal education was coded as low (no diploma or primary school), middle (high school diploma), and high (college or university degree). Smoking status was classified as non-smokers, stopped smoking before pregnancy, and current smokers (smoked during pregnancy). Alcohol use was subdivided in mothers who did not consume alcohol during pregnancy and mothers who consumed alcohol at least occasionally during gestation. The follow-up examinations in this study population were performed between October 3, 2014, and July 12, 2018. Within this timeframe, 587 children were between 4 and

6 years old and could hence participate in the first follow-up step of ENVIR*ON*AGE. Thirteen mother-child pairs were not eligible for participation since their child had passed away (n = 1) or they had moved to another country or too far from the location where the examination took place (n = 12). Of the remaining 574 mother-child pairs, 74 people could not be contacted by e-mail or phone since no up-to-date contact details could be retrieved, three mother-child pairs could not be contacted at the moment that the child had an age between 4 and 6, 165 women refused to participate, and 332 renewed consent (i.e., a final participation rate of 58%) (**Supplemental Figure S1**). A second questionnaire was filled out by the participants on the day of the follow-up examination, which provided us with information on lifestyle conditions of the child, for example on the passive exposure to parental smoking. Passive smoking status was classified as not exposed, exposed by one parent, or exposed via smoking of both parents.

Clinical measurements

The clinical investigation of the retinal blood vessels and blood pressure were performed by a single trained examiner, following standardized guidelines. Children always gave their assent before measurements were initiated. The blood pressure of the participants was measured with an automated oscillometric upper-arm blood-pressure monitor (Omron 705IT, Omron Corporation, Japan). To ensure accurate measurements, cuffs adapted for children were used depending on the child's right upper arm circumference. Measurements were performed by a standardized method, as described by the European Society of Hypertension.[195] In summary, after the child had rested for ten minutes in supine position, a trained observer obtained five consecutive readings of the systolic (SBP) and diastolic (DBP) blood pressure of the right arm, with one-minute intervals. Average SBP and DBP were based on the last three readings. Mean arterial pressure (MAP) was calculated via the equation: MAP = (2/3 x DBP) + (1/3 x SBP).

We used retinal vascular imaging as a proxy to examine the systemic microvasculature between the age of four and six. This approach has been proven successful in other studies that examined early lifestyle factors related to cardiovascular disease.[196] To determine the retinal blood vessel parameters, fundus pictures of the oculus dextrus and oculus sinister were taken with a Canon

CR-2 plus 45° 6.3 megapixel digital nonmydriatic retinal camera (Hospithera, Brussels, Belgium). These pictures were subsequently analyzed with the MONA-REVA® software (version 2.1.1, VITO Health, Mol, Belgium). First, the diameter of the optic disc (OD) was determined for each picture, since all distance measurements within the fundus were set relative to this value. Next, the widths of the retinal arterioles and venules were calculated within the predetermined area of 0.5 and 1 times the OD diameter, starting from the margin of the optic disc (**Supplemental Figure S2**). The diameters of the six largest arterioles and six largest venules in this zone were used in the revised Parr-Hubbard formula to calculate the central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE).[197] The tortuosity index (TI) of the retinal vasculature was determined between 1.5 and 5 times the radius of the OD and can be described as a measure for the curvature of the retinal vessels in this zone. The normalized tortuosity is calculated as the average tortuosity of the branch segments, where the tortuosity of a branch segment is the ratio of the line traced on each tree along the vessel axis between 0.5 and 2 times the OD diameter and the line connecting the endpoints. Segmentations are cropped centered on the OD whereby the inner and outer radii were taken at 1.5 and 5.0 times the radius of the OD.[198]

Of the 332 mother-child pairs, 74 were not included in the statistical analyses since no (good quality) images of the retinal vasculature of either one eye were available (**Supplemental Figure S1**). Pictures could not be taken due to the participant having autism spectrum disorder (n = 2), severe mental retardation (n = 1), blindness (n = 1), and lack of concentration or unwillingness to participate (n = 20). For 50 participants, the quality of the pictures was suboptimal, due to children's movement of their body or eyes during the capturing of the images. Of the remaining 258 mother-child pairs, the data on alcohol use during pregnancy were missing for three mothers, and for ten children, no blood pressure could be determined at the moment of the follow-up visit. Therefore, these 13 participants were excluded, and final statistics were performed on 245 mother-child pairs.

Exposure assessment

The maternal residential address was used to interpolate regional background levels of $PM_{2.5}$ and NO_2 concentrations (µg/m³) during pregnancy and for the postnatal period, based on a high-resolution spatial-temporal interpolation method [103], as described in the cohort profile paper of Janssen *et al.* The overall model performance was assessed by leave-one-out cross-validation, including 44 monitoring stations for NO_2 and 34 stations for $PM_{2.5}$. The validation statistics of the interpolation tool explained more than 78% of the temporal and spatial variability in Flanders for NO_2 and 80% for $PM_{2.5}$.[104,156] Furthermore, accuracy of our exposure models was recently proven by the correlation between the urinary load of nano-sized black carbon particles in children and residential levels of $PM_{2.5}$ and NO_2 [106], and for prenatal exposure by a correlation between this exposure model and the placental black carbon load.[34] The described model provided daily air pollution levels for each participant. Daily values were averaged for each specific time window during the pregnancy: first trimester (i.e., date of conception until 13 weeks of pregnancy), second trimester (i.e., 14 weeks until 26 weeks of pregnancy), third trimester (i.e., 27 weeks of pregnancy until delivery), and the entire period of pregnancy, from the date of conception until the date of delivery.

Ultrasound imaging data combined with the first day of the mother's last menstrual period were used to estimate the date of conception.[44] Postnatal $PM_{2.5}$ and NO_2 exposures were averaged for the day of the follow-up visit, the day before the follow-up visit, and the week before the follow-up visit as short-term exposure windows, and the average exposure during childhood (i.e., the average daily exposure from the day of birth until the day before the follow-up visit) as long-term exposure window. Exposure data during childhood were available for 226 out of the 245 participants. For mothers who moved during pregnancy (n = 21) and between birth and follow-up examination (n = 69), we calculated the specific exposures, allowing for the changes in the corresponding period.

Statistics

We used SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA) for data analysis. Normality and equality of variances, as assumptions of model linearity, were tested with the Shapiro-Wilk statistic and Q-Q plots of the residuals, respectively. Differences in characteristics between participants and non-participants of the follow-up study were tested by means of a two-sided *t* test for continuous variables and with a Chi-Square test for the categorical variables. Unadjusted correlations between prenatal exposure to $PM_{2.5}$ and NO_2, and the retinal microvascular characteristics, as well as between prenatal and postnatal air pollution levels, were examined by means of Pearson's correlation. In the main analyses, the association of retinal vessel widths (CRAE and CRVE) and vessel curvature (TI) with either prenatal or postnatal $PM_{2.5}$ and NO_2 exposure was assessed using multivariable linear regression modeling. Pregnancy trimester-averaged $PM_{2.5}$ and NO_2 exposure levels were entered into the same model in order to estimate independent trimester-specific effects. Minimally adjusted models were adjusted for sex and age (years), and we adjusted the analyses for the following variables: age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), the age of the mother during pregnancy and her pre-pregnancy BMI, the education level of the mother, alcohol use of the mother during pregnancy, the smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. The interaction between whole pregnancy air pollution exposure and age, sex, MAP, and BMI on the microvascular parameters was explored using continuous variables. We found a significant interaction for MAP and prenatal NO_2 exposure on CRAE and CRVE; therefore, we additionally constructed a variable indicating high and low MAP (based on the median) and stratified the analysis accordingly. In the secondary analyses, we combined entire pregnancy exposure and postnatal exposures during either the day of the follow-up examination, the week before the followup visit, or the entire childhood exposure in the same model. The magnitude of all associations was expressed for an interquartile range (IQR; between the 25th and 75th percentile) increase in the observed exposure.

We performed three separate sensitivity analyses. Firstly, a sensitivity analysis was conducted to assess the association between prenatal air pollution exposure and the CRVE, CRAE and TI in a population excluding mothers with diagnosed hypertension during pregnancy (n = 8) and those with gestational diabetes (n = 15). Furthermore, Wei and colleagues found that prematurity could also affect the retinal vessel characteristics of children later in life.[199] Therefore, we performed an additional sensitivity analysis excluding the children who were born before 37 weeks of gestation (n = 12). Finally, a third sensitivity analysis was conducted, excluding mothers who smoked during pregnancy (n = 32).

RESULTS

Study population characteristics

Background characteristics of the 242 non-participants were similar to those of the participants with analysed retinal images and full data (n = 245) and the participants of the follow-up study with poor quality pictures or without full data (n = 87) with respect to parity, pre-pregnancy BMI and smoking behavior, child's sex distribution, birth weight and birth length (**Supplemental Table S1**). However, mothers that renewed consent were significantly older at the birth of their child, and were more likely to have a higher education level and to be of European ancestry compared with non-participants. Prenatal levels of residential particulate air pollution by trimester did not differ, while the total gestational exposure was on average slightly higher in participants compared with non-participants. For prenatal NO_2 none of the exposure windows showed difference between participants and non-participants. The postnatal $PM_{2.5}$ and NO_2 exposure did not differ between the three groups.

The characteristics of the 245 mother-child pairs participating in this study are summarized in **Table 1**. At the time of birth, the mothers had an average [standard deviation] age of 29.9 [4.1] years. Their pre-pregnancy BMI was 24.4 [4.6] kg/m². Most of the mothers had never smoked (n = 167; 68.2%) or stopped smoking before pregnancy (n = 46; 18.8%) and 78.8% of the mothers did not consume alcohol throughout their pregnancy. Almost three-quarters of all women

were highly educated (67.4%), having a college degree or higher.

At the moment of the follow-up examination, the children (52.6% girls) had an average age of 4.6 [0.4] years, an average height of 107.3 [4.9] cm and an average weight of 18.7 [2.7] kg. Mean arterial pressure averaged 68.5 [6.0] mmHg. Most of the follow-up visits took place during spring (33.9%) and winter (29.4%). Most children were not exposed to passive smoking in their home environment (n = 170), while 18.8% of the children were exposed via one parent and 11.8% of the children were exposed to passive smoking via both parents.

Exposure characteristics

Table 2 summarizes the $PM_{2.5}$ and NO_2 exposure characteristics of the study population during pregnancy and early childhood. The average exposures for both air pollution components were comparable between the three trimesters and the entire pregnancy. For $PM_{2.5}$, an average exposure (IQR) of 14.3 (12.6 – 15.8) μg/m³ was calculated for the entire pregnancy, and for the same period, the average NO_2 level was 19.7 (16.5 – 22.7) μg/m³. The average (IQR) childhood exposure level calculated for $PM_{2.5}$ was 12.6 (11.9 – 13.3) μg/m³, while for NO_2, this was 17.2 (14.6 – 19.3) μg/m³. All $PM_{2.5}$ and NO_2 exposures of the three trimesters and whole pregnancy were strongly correlated ($p \leq 0.02$), except for the first and third trimester NO_2 ($r = -0.07$, $p = 0.29$), third trimester NO_2 and second trimester $PM_{2.5}$ ($r = 0.03$, $p = 0.65$), and first trimester $PM_{2.5}$ and whole pregnancy NO_2 exposure ($r = -0.04$, $p = 0.51$). A strong correlation was identified for exposure during the entire pregnancy and lifetime of the children, both for $PM_{2.5}$ ($r = 0.32$, $p < 0.0001$) and NO_2 exposures ($r = 0.81$, $p < 0.0001$).

Table 1. Average (SD) or numbers (%) of the characteristics of the mother-child pairs included in this study (n = 245), and the girls (n = 129) and boys (n = 116) separately. The p-value depicts the difference between girls and boys.

Characteristic	Combined	Girls	Boys	p-value
Mother				
Age at birth child, years	29.9 (4.1)	30.1 (4.1)	29.7 (4.1)	0.41
Pre-pregnancy BMI, kg/m²	24.4 (4.6)	24.4 (4.9)	24.3 (4.3)	0.78
Smoking behavior during pregnancy				0.48
Never smoked	167 (68.2)	90 (36.8)	77 (31.4)	
Stopped smoking before pregnancy	46 (18.8)	24 (9.8)	22 (9.0)	
Smoked during pregnancy	32 (13.0)	15 (6.1)	17 (6.9)	
Alcohol consumption during pregnancy				0.67
Yes	52 (21.2)	26 (10.6)	26 (10.6)	
No	193 (78.8)	103 (42.1)	90 (36.7)	

Continued

Table 1. **Continued**

Characteristic	Combined	Girls	Boys	p-value
Education level				0.59
Low (no high school diploma)	16 (6.5)	7 (2.9)	9 (3.7)	
Middle (high school diploma)	64 (26.1)	34 (13.9)	30 (12.2)	
High (college degree or higher)	165 (67.4)	88 (35.9)	77 (31.4)	
Child				
Ethnicity				0.26
European	230 (93.9)	119 (48.6)	111 (45.3)	
Non-European	15 (6.1)	10 (4.1)	5 (2.0)	
Birth weight, g	3446.6 (429.8)	3413.3 (412.9)	3483.5 (446.8)	0.20
Age at time of follow-up, years	4.6 (0.4)	4.6 (0.4)	4.5 (0.4)	0.75
Height at time of follow-up, cm	107.3 (4.9)	107.9 (5.1)	107.8 (4.6)	0.81

Continued

Table 1. **Continued**

Characteristic	Combined	Girls	Boys	p-value
Weight at time of follow-up, kg	18.7 (2.7)	18.8 (2.7)	18.7 (2.7)	0.70
BMI at time of follow-up, kg/m²	16.0 (1.4)	16.1 (1.4)	16.0 (1.4)	0.68
Season at follow-up				0.82
Spring	83 (33.9)	46 (18.8)	37 (15.1)	
Summer	48 (19.6)	25 (10.2)	23 (9.4)	
Autumn	42 (17.1)	18 (7.3)	24 (9.8)	
Winter	72 (29.4)	40 (16.3)	32 (13.1)	
Systolic blood pressure, mmHg	97.6 (8.2)	98.1 (8.0)	97.0 (8.5)	0.30
Diastolic blood pressure, mmHg	53.9 (6.9)	54.5 (6.6)	53.2 (7.1)	0.13
Mean arterial pressure, mmHg	68.5 (6.0)	69.1 (5.6)	67.8 (6.3)	0.10

Continued

Table 1. Continued

Characteristic	Combined	Girls	Boys	p-value
Exposure to passive smoking				0.43
Not exposed	170 (69.4)	88 (35.9)	82 (33.5)	
Exposed via one parent	46 (18.8)	23 (9.4)	23 (9.4)	
Exposed via both parents	29 (11.8)	18 (7.3)	11 (4.5)	
CRAE, μm	180.8 (14.2)	182.5 (13.0)	178.9 (15.3)	0.05
CRVE, μm	251.0 (19.4)	253.7 (18.1)	247.9 (20.5)	0.02
TI	0.889 (0.012)	0.891 (0.012)	0.887 (0.013)	0.04

Abbreviations: CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; TI, Tortuosity index; SD, Standard deviation.

Table 2. **Exposure details on $PM_{2.5}$ and NO_2 air pollution (µg/m³) during different time windows of pregnancy and different periods during the childhood of the participants.**

Time window of pregnancy	Mean (SD)	25th percentile	75th percentile	IQR
$PM_{2.5}$				
Pregnancy				
Trimester 1	14.3 (5.5)	9.6	18.2	8.6
Trimester 2	14.2 (5.1)	9.6	17.9	8.3
Trimester 3	14.2 (5.7)	9.1	18.3	9.2
Entire pregnancy	14.3 (2.3)	12.6	15.8	3.2
Childhood				
Day of follow-up visit	11.2 (8.3)	4.8	15.1	10.3
Day before follow-up visit	12.1 (10.2)	4.5	15.9	11.4
Week before follow-up visit	13.0 (7.5)	7.3	17.7	10.4
Average childhood exposure	12.6 (1.1)	11.9	13.3	1.4

Continued

Table 2. **Continued.**

Time window of pregnancy	Mean (SD)	25th percentile	75th percentile	IQR
NO_2				
Pregnancy				
Trimester 1	19.9 (6.0)	15.2	24.3	9.1
Trimester 2	19.8 (6.2)	15.0	24.1	9.1
Trimester 3	19.6 (6.2)	14.7	23.7	9.0
Entire pregnancy	19.7 (4.4)	16.5	22.7	6.2
Childhood				
Day of follow-up visit	17.0 (8.7)	10.5	21.3	10.8
Day before follow-up visit	17.2 (9.5)	9.7	22.8	13.1
Week before follow-up visit	17.2 (7.2)	11.7	21.7	10.0
Average childhood exposure	17.2 (3.4)	14.6	19.3	4.7

Abbreviations: IQR, interquartile range; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; SD, standard deviation

Microvasculature characteristics

The average CRAE, CRVE, and TI values of both eyes was used for each child if both pictures were available. For 200 participants the retina pictures of both eyes were used; for 27 individuals only that of the left eye and for 18 children only the picture of the right eye was available for analysis since the picture of the other eye was of insufficient quality. There was no difference between the values of either one or two eyes for the CRAE ($p = 0.38$), CRVE ($p = 0.38$) or TI ($p = 0.38$).

For all children, the average [SD] CRAE and CRVE were 180.8 [14.2] µm and 251.0 [19.4] µm respectively, and the average TI was 0.889 [0.012] (**Table 1**). The CRAE, CRVE and TI were slightly higher in girls than in boys (**Table 1**). A positive correlation was found between both CRVE and CRAE ($r = 0.60$, $p < 0.0001$), between CRVE and TI ($r = 0.19$, $p = 0.003$), and between CRAE and TI ($r = 0.14$, $p = 0.02$).

Main analyses

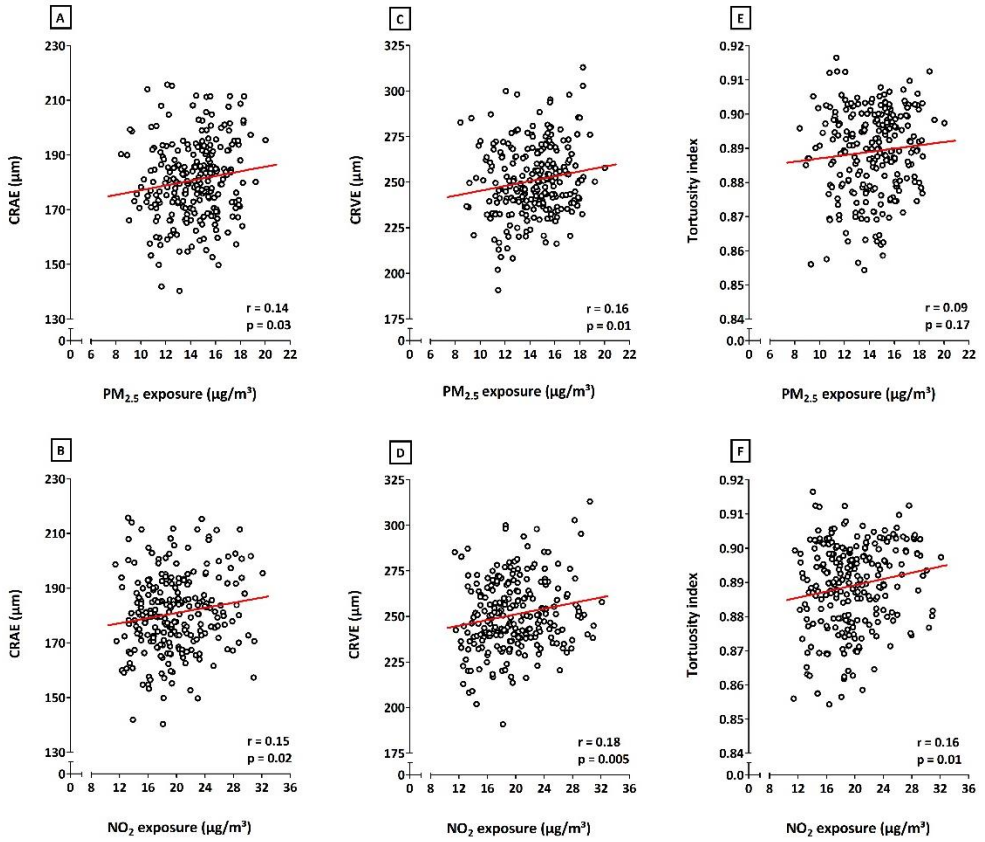
Associations between prenatal air pollution exposure and retinal microvasculature

Correlations analyses, without additional adjustments, showed positive relationships between *in utero* exposure to both $PM_{2.5}$ and NO_2 during the entire pregnancy and the CRAE, CRVE, and TI (**Figure 1**). Only the association between entire pregnancy exposure to $PM_{2.5}$ and the tortuosity of the retinal vessels was not significant ($p \leq 0.05$). Correlations between microvascular traits and $PM_{2.5}$ or NO_2 exposure during the separate trimesters of pregnancy were only significant between third trimester NO_2 exposure and TI (data not shown).

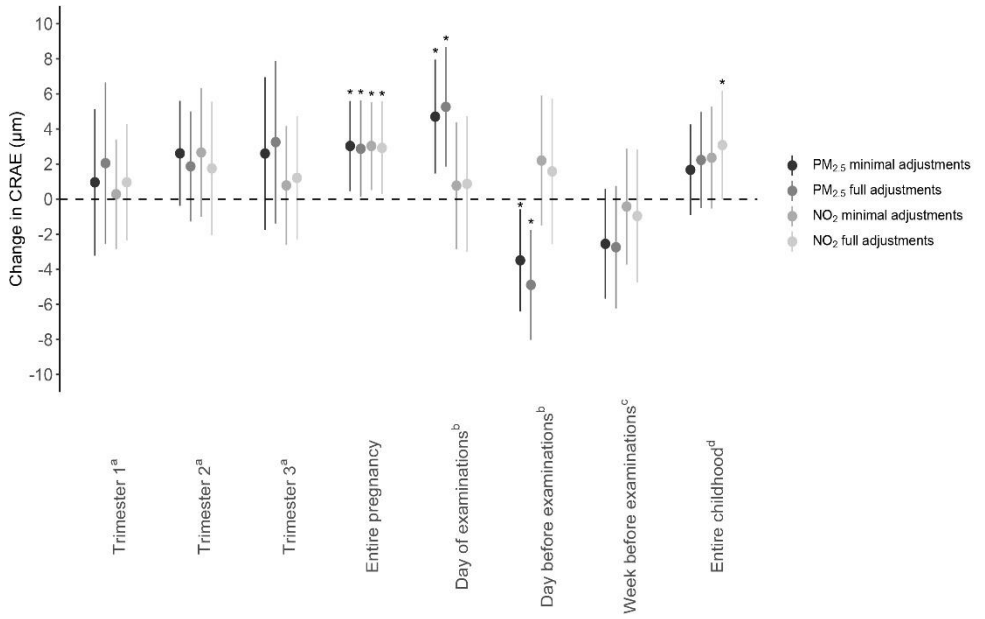
Multiple linear regression modeling showed a positive association between entire pregnancy exposure to $PM_{2.5}$ and CRAE (**Figure 2**). With full adjustments, for every IQR increase in $PM_{2.5}$ air pollution exposure during pregnancy, a 2.87 µm widening of the arteriolar diameter was observed (95% CI: 0.12 to 5.62; $p = 0.04$). For whole pregnancy exposure to NO_2, a widening of 2.92 µm of the arterial diameter was determined for every IQR increase in exposure (95% CI: 0.29 to

5.56; p = 0.03). No significant changes in CRAE were observed for increased levels of either NO_2 or $PM_{2.5}$ exposure during the three separate trimesters of pregnancy. An IQR increment in prenatal air pollution exposure during the entire pregnancy was associated with a 3.85 µm (95% CI: 0.10 to 7.60; p = 0.04) higher CRVE for $PM_{2.5}$ and a 4.03 µm (95% CI: 0.44 to 7.63; p = 0.03) widening of the retinal venules for NO_2 exposure in the fully adjusted models (**Figure 3**). Again, as observed for the CRAE, no significant changes in CRVE were determined in association with trimester-specific exposure. $PM_{2.5}$ exposure during pregnancy was not associated with the tortuosity index. However, an association between TI and *in utero* NO_2 exposure was found in both minimally and fully adjusted models. In fully adjusted models, an IQR increase in prenatal NO_2 exposure over the entire pregnancy was associated with a 0.0028 (95% CI, 0.0005 to 0.0051, p = 0.02) higher TI, which was mainly driven by the exposure in the third trimester (**Figure 4**).

With same full adjustments as before, we explored the interaction between age, sex, MAP and BMI, and microvascular parameters using continuous variables. These interaction terms were all non-significant ($p \geq 0.18$) except borderline significance for mean arterial pressure in association with both CRAE and CRVE for whole pregnancy NO_2 exposure. Categorizing MAP above and below the median revealed stronger prenatal NO_2 exposure effects on CRAE and CRVE associations in children in the high MAP group (**Supplemental Table S2**).



<u>Figure 1.</u> Correlations between exposure to either $PM_{2.5}$ (first row) or NO_2 (second row) during the entire pregnancy and CRAE (A and B), CRVE (C and D) and the Tortuosity Index (E and F). The respective r- and p-values are depicted on each plot. Abbreviations: CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm



<u>Figure 2.</u> Associations between CRAE and $PM_{2.5}$ or NO_2 exposure during pregnancy or during childhood. Estimates are given as change (95% CI) for every IQR increase in $PM_{2.5}$ (two darker grey dots) or NO_2 (two lighter grey dots). Minimally adjusted models were adjusted for sex and age (years), fully adjusted models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. [a] Model adjusted for the three pregnancy trimester-averaged exposures levels, [b] Model adjusted for exposure on the day of the follow-up visit and exposure of the day preceding the follow-up visit, [c] Model adjusted for exposure on the day of the follow-up visit and exposure of the week preceding the follow-up visit, [d] Model adjusted for exposure on the day of the follow-up visit and average childhood exposure from the day of birth until the day before the follow-up examination. Abbreviations: CI, confidence interval; CRAE, Central retinal arteriolar equivalent; IQR, Interquartile range; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm. *$p \leq 0.05$.

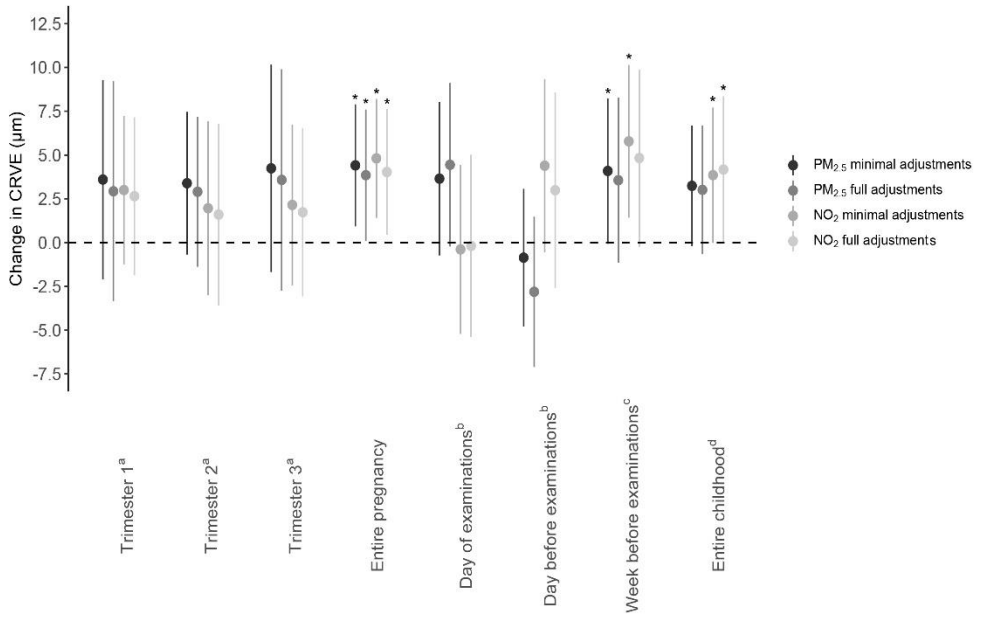


Figure 3. Associations between CRVE and $PM_{2.5}$ or NO_2 exposure during pregnancy or during childhood. Estimates are given as change (95% CI) for every IQR increase in $PM_{2.5}$ (two darker grey dots) or NO_2 (two lighter grey dots). Minimally adjusted models were adjusted for sex and age (years), fully adjusted models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. [a] Model adjusted for the three pregnancy trimester-averaged exposures levels, [b] Model adjusted for exposure on the day of the follow-up visit and exposure of the day preceding the follow-up visit, [c] Model adjusted for exposure on the day of the follow-up visit and exposure of the week preceding the follow-up visit, [d] Model adjusted for exposure on the day of the follow-up visit and average childhood exposure from the day of birth until the day before the follow-up examination. Abbreviations: CI, confidence interval; CRVE, Central retinal venular equivalent; IQR, Interquartile range; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm. *$p \leq 0.05$

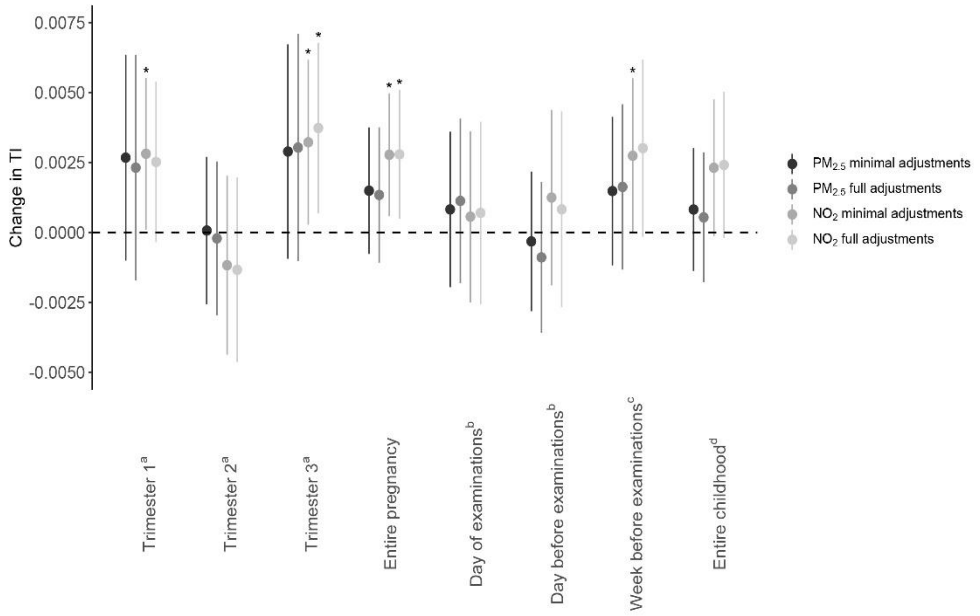


Figure 4. Associations between TI and $PM_{2.5}$ or NO_2 exposure during pregnancy or during childhood. Estimates are given as change (95% CI) for every IQR increase in $PM_{2.5}$ (two darker grey dots) or NO_2 (two lighter grey dots). Minimally adjusted models were adjusted for sex and age (years), fully adjusted models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. [a] Model adjusted for the three pregnancy trimester-averaged exposures levels, [b] Model adjusted for exposure on the day of the follow-up visit and exposure of the day preceding the follow-up visit, [c] Model adjusted for exposure on the day of the follow-up visit and exposure of the week preceding the follow-up visit, [d] Model adjusted for exposure on the day of the follow-up visit and average childhood exposure from the day of birth until the day before the follow-up examination. Abbreviations: CI, confidence interval; IQR, interquartile range; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; TI, Tortuosity index. *p ≤ 0.05

Associations between postnatal $PM_{2.5}$ and NO_2 exposure and retinal microvasculature

Exposure to $PM_{2.5}$ on the day of the follow-up examination and the day before the visit had a significant effect on the CRAE (**Figure 2**) but not on the CRVE ($p \geq 0.10$) (**Figure 3**). The arterial diameter was 5.26 µm wider (95% CI: 1.86 to 8.67, $p = 0.003$) for every IQR increase in $PM_{2.5}$ exposure on the day of the measurements, while a narrowing of 4.89 µm (95% CI: -8.02 to -1.76, $p = 0.002$) was determined for every IQR higher $PM_{2.5}$ exposure on the day before the follow-up visit. No associations were found between NO_2 exposure and either CRAE, CRVE or TI for exposure on the day on which, or the day before, the retinal images were taken. When the exposure of the week before the follow-up examination was considered an association between NO_2 exposure and CRVE could be identified in the minimally adjusted model, which disappeared following full adjustment (**Figure 3**). In case of average exposure during the lifetime of the children, in fully adjusted models, we observed a 3.08 µm (95% CI: -0.01 to 6.18, $p = 0.05$) widening of the CRAE (**Figure 2**) and a 4.17 µm (95% CI: -0.01 to 8.35, $p = 0.05$) widening of the CRVE (**Figure 3**) for every IQR increase in NO_2 during the child's lifetime.

Secondary analyses

Associations between retinal microvasculature and residential air pollution in combined prenatal and postnatal exposure models

In secondary analyses, we combined prenatal exposure during the entire pregnancy and postnatal exposure to either $PM_{2.5}$ or NO_2 in one model. NO_2 exposure during the entire gestation was correlated with both short-term exposure (day of the follow-up, day before the follow-up, and week before follow-up; $r = 0.35$, $r = 0.34$, and $r = 0.45$ respectively, $p < 0.0001$) and long-term exposure (entire childhood; $r = 0.81$, $p < 0.0001$) to ambient NO_2. For $PM_{2.5}$, we only noted a correlation between entire pregnancy exposure and postnatal exposure during the entire childhood ($r = 0.32$, $p < 0.0001$).

Only the associations between postnatal exposure to $PM_{2.5}$ on the day of, or the day before, the follow-up visit and CRAE remained significant ($p = 0.003$) in models mutually adjusted for exposure during the entire pregnancy (results not shown). However, adjusting modeled entire pregnancy exposure for short-term postnatal exposure (i.e., on the day of the follow-up, or on the day or the week before the examination) increased the effect estimates for both the CRAE and CRVE, while the estimates for the TI did not substantially change. Moreover, the associations with entire pregnancy exposure and the retinal vessel diameter and tortuosity remained, except for CRVE and TI after adjustment of the model for NO_2 exposure during the day or in the week before the follow-up examination (**Table 3**). Including exposure during the entire childhood into the model decreased the estimates of both the CRVE and TI in association with every IQR increase in whole pregnancy $PM_{2.5}$ and NO_2 exposure and that of the CRAE in association with prenatal NO_2, while a higher estimate for the CRAE was found in association with increased prenatal $PM_{2.5}$ exposure after mutual adjustment for entire childhood $PM_{2.5}$ exposure. Only the latter association remained significant in the model including whole postnatal air pollution exposure (**Table 3**).

Table 3. Associations between retinal microvascular characteristics and $PM_{2.5}$ or NO_2 exposure during pregnancy: results from secondary analyses with models combining entire pregnancy exposure with different postnatal exposure periods.

Model	CRAE, µm	CRVE, µm	TI
$PM_{2.5}$			
Entire pregnancy + Day of follow-up	3.76 (0.76 to 6.75)*	4.29 (0.24 to 8.33)*	0.0022 (-0.0003 to 0.0047)
Entire pregnancy + Day before follow-up	3.75 (0.75 to 6.74)*	4.32 (0.26 to 8.39)*	0.0022 (-0.0003 to 0.0048)
Entire pregnancy + Week before follow-up	3.73 (0.71 to 6.74)*	4.71 (0.68 to 8.74)*	0.0024 (-0.0002 to 0.0049)
Entire pregnancy + Entire childhood	3.25 (0.13 to 6.37)*	3.53 (-0.69 to 7.74)	0.0022 (-0.0005 to 0.0048)
NO_2			
Entire pregnancy + Day of follow-up	2.96 (0.05 to 5.87)*	4.24 (0.32 to 8.16)*	0.0027 (0.0002 to 0.0051)*
Entire pregnancy + Day before follow-up	2.89 (-0.01 to 5.79)*	3.90 (-0.01 to 7.81)*	0.0027 (0.0002 to 0.0051)*
Entire pregnancy + Week before follow-up	3.83 (0.74 to 6.91)*	3.25 (-0.90 to 7.40)	0.0021 (-0.0005 to 0.0047)
Entire pregnancy + Entire childhood	1.68 (-3.22 to 6.58)	3.09 (-3.51 to 9.70)	0.0024 (-0.0017 to 0.0065)

Estimates are given as change (95% CI) per IQR increase in exposure to either $PM_{2.5}$ or NO_2 during the entire pregnancy. All models were adjusted for exposure to either $PM_{2.5}$ or NO_2 during the entire pregnancy, age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. The separate models were additionally adjusted for exposure during either the day of the follow-up visit, the day before the follow-up examination, the week preceding the follow-up visit or the average childhood exposure from the day of birth until the day of the follow-up examination. Abbreviations: CI, confidence interval; CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; TI, Tortuosity index. *$p \leq 0.05$

Sensitivity analyses

Excluding mothers who were diagnosed with hypertension (n = 8) and gestational diabetes (n = 15) during pregnancy slightly decreased the reported estimates of all three retinal vessel characteristics, in association with both of the examined whole pregnancy exposures (**Supplemental Table S3**). The associations between the entire pregnancy $PM_{2.5}$ exposure and both CRVE and CRAE, and between NO_2 exposure during the entire gestation and CRAE lost their significance ($p > 0.06$). Similar findings could be concluded for the sensitivity analysis excluding newborns with a gestational age lower than 37 weeks (n = 12) (**Supplemental Table S4**). However, the association between entire pregnancy NO_2 exposure and CRAE remained significant in this subgroup. Finally, a third sensitivity analysis excluding mothers who had smoked during pregnancy (n = 30) did not substantially alter the described relationships between the retinal vessel characteristics and either $PM_{2.5}$ or NO_2 exposure during pregnancy (**Supplemental Table S5**).

DISCUSSION

We have evaluated the associations between gestational and childhood exposure to ambient air pollution and microvascular structure by using retinal vessel metrics in four- to six-year-old children. The key findings are as follows: 1) retinal venular and arterial diameters of children widen with a higher exposure of their mother to $PM_{2.5}$ and NO_2 during the entire pregnancy period, and 2) the retinal blood vessel curvature is affected by *in utero* exposure to NO_2, represented by an increase in tortuosity index for the entire pregnancy and the third trimester. To our knowledge, we are the first to find an association between air pollution exposure during gestation and effects on the retinal microvasculature later in life.

Several studies found associations between retinal vascular characteristics and both acute and chronic exposure to air pollution in middle-aged or older populations. One of these studies was performed in a population of healthy adults with an average age of 64 from the Multi-Ethnic Study of Atherosclerosis (MESA) cohort study.[191] In this group, narrower retinal arterioles and wider venules were

observed with increased 2-year exposure to $PM_{2.5}$. A Belgian study focused on the effects of short-term air pollution exposure on retinal microcirculation in adults between the age of 22 and 63. Both the average CRAE and CRVE in this research decreased with increasing exposure to PM_{10} and black carbon.[200] These results were further supported and even associated with changes in specific miRNA's linked to inflammatory and oxidative stress pathways.[201] Provost *et al.* were the first to describe the relationship between retinal vessel diameter and both short-term and long-term exposure to $PM_{2.5}$ air pollution in children.[82] They determined exposures on the day of the retinal examinations, as well as in the year prior to their measurements. In accordance with our results, the authors found that there was a more significant effect of short-term exposure on the CRAE, in contrast to the CRVE. For long-term average annual exposure, no effects could be observed on both the CRAE and CRVE in this population in 8-to-12-year-old children.

Although studies focus on different forms and time windows of ambient air pollution exposure during the lifetime, there is a clear indication that the diameter of retinal venules is affected. Several systemic and environmental factors have been attributed to a wider CRVE over the course of life. For example, retinal venular widening has not only been associated with the effects of active smoking but also with systemic diseases such as diabetes and obesity.[202,203] Research conducted within the Rotterdam Study, in a population of people over the age of 55, showed that both the venular and arteriolar retinal diameters widened when the participants had formerly smoked or were active smokers, with the largest effect on the retinal venules.[204] Widening of the retinal venules has also been considered as a potential biomarker for adverse health conditions. A meta-analysis combining the results of six individual prospective cohort studies has shown that a wider CRVE can be an indicator of coronary heart disease in adult women.[205] Furthermore, a recent long-term follow-up cohort study has described that the width of retinal venules could be a potential predictor of ischemic stroke in both men and women and, in accordance with the former meta-analysis, of coronary heart disease in women.[190]

In this research, we have described a positive association between CRAE and prenatal exposure to $PM_{2.5}$ and NO_2. In the context of exposure to air pollution, the changes in CRAE mostly seem to show negative relationships between the exposure variable and retinal arterial diameter.[191,200,201] Indeed, a narrower CRAE has been associated with several detrimental cardiovascular health outcomes, such as hypertension and arterial stiffness.[206] However, environmental exposures or adverse conditions associated with an increase of the CRAE have also been described over the past years. A wider CRAE was linked with high cholesterol in a population of the Locomotive Syndrome and Health Outcome in Aizu Cohort Study and with cigarette smoking in the MultiEthnic Study of Atherosclerosis (MESA) study. Potential mechanisms explaining these effects are endothelial dysfunction and elevated oxidative stress as observed in mouse models.[207] A widening of the retinal arterial diameter has also been associated with several disease outcomes. Rhee *et al.* found that people who were diagnosed with intracranial arterial stenosis had a higher CRAE compared to those without the condition [208], while another study conducted within an Asian population showed an association between wider CRAE values and a higher incidence of diabetes.[209] Since both narrowing and widening of the retinal arterioles have been associated with detrimental health outcomes later in life, our findings should be traced further within the follow-up cohort, to be able to evaluate the changes in CRAE and the correlated health changes in these children throughout their life course.

Not only the diameter of the retinal vessels was described to be affected by exposure to air pollution *in utero*. In this study, the tortuosity of the vessel network was found to increase with higher exposure to NO_2 during the entire period of pregnancy. Tortuosity can be regarded as a measure for vessel curvature and has been found to be influenced by conditions such as diabetes and hypertension.[210] Although this microvascular characteristic has been studied to a lesser extent than the retinal vascular diameter, with an apparent lack of studies on the relation to environmental exposures, vessel tortuosity has also been identified as a potential marker for the risk of developing cardiovascular disease. For example, higher microvascular tortuosity in the retina has been associated with an increased risk of developing cerebral microbleeds [211] and ischemic stroke.[212]

The World Health Organization (WHO) and European limits that have been determined on the short-term (1-hour mean) and long-term (annual mean) exposure to NO_2 are 200 and 40 µg/m³ respectively.[213] However, the effects described in this research have been determined for a mean exposure of 19.7 µg/m³ NO_2 over the entire pregnancy, which is merely half of the WHO annual guideline value. A recent meta-analysis has shown that exposure to increased levels of NO_2 augments both respiratory and cardiovascular mortality and is in itself, apart from $PM_{2.5}$, an important catalyst in disease development and even mortality.[32] These results thus show that the effects of NO_2 exposure on the (micro-) circulation cannot be underestimated and should be further studied in terms of the effect of prenatal exposures on development later in life.

We acknowledge that this study has several strengths and limitations. A first strength is that this project is the first of its kind, investigating the effects of environmental $PM_{2.5}$ and NO_2 air pollution exposure occurring before birth, and during the child's lifetime in both short- and long-term periods, on the microvasculature later in life. These results originate from a prospective birth cohort study and thanks to extensive bio-banking, data collection at birth and at follow-up examination, we were able to give a very precise estimation of the effect of exposure to air pollution on retinal vessel characteristics at the age of four to six. In this way, we contribute to the field of knowledge studying the complex relationships between prenatal and postnatal environmental exposures and (disease) development later in life. Secondly, we used data from a large population-based sample size of children, representative for the reproductive segment of the Flemish population of Belgium.[88] A third strength of this study is that retinal image analysis has been performed by one researcher blinded for the exposure data, excluding examiner bias. A limitation of this study is that, although the confounding factors in our statistical model were selected following an *a priori* thorough examination, residual confounding posed by other environmental factors or population characteristics cannot be fully excluded. Another limitation is the potential misclassification of exposure. Our results are based on daily levels of residential particulate exposure during prenatal and postnatal life but do not account for exposures other than residential. However, the accuracy of our exposure models and relevance for personal and internal exposure has been

proven, since air pollution levels at the residential address and proxies thereof, such as proximity of the home to major roads, correlates with the levels of nano-sized black carbon levels measured in the urine of children living in the same study area.[106]

CONCLUSIONS

Both prenatal and early childhood exposures to $PM_{2.5}$ and NO_2 were associated with changes in retinal vessel diameters and altered vessel tortuosity in young children. Our study adds to the knowledge of basic fundamental mechanisms on the complex relationship between early life exposure to ambient air pollution and cardiovascular disease development later in life. In future research projects, focus should be put on the implications of our findings on the cardiovascular development of the children in our prospective cohort.

SUPPLEMENTAL MATERIAL

Supplemental Table S1. Comparison of characteristics of the eligible mother-child pairs who participated in the follow-up study and had analyzed retinal pictures and full data (n = 245) or had poor quality pictures or missing data (n = 87) or did not participate in the follow-up study of the ENVIR*ON*AGE birth cohort (n = 242).

Characteristics	Participants follow-up with analysed retina pictures and full data (n = 245)	Participants follow-up with poor quality retina pictures and/or missing data (n = 87)	Non-participants follow-up (n = 242)	Overall p-value
Mother				
Age at birth child, years	29.9 (4.1)[a]	30.5 (4.6) [b]	27.9 (4.9) [a,b]	<0.0001
Pre-pregnancy BMI, kg/m²	24.4 (4.6)	23.6 (4.2) [a]	24.8 (4.8) [a]	0.10
Parity				0.84
1	125 (51.0)	51 (58.6)	128 (52.9)	
2	92 (37.6)	25 (28.7)	90 (37.2)	
3 ≤	28 (11.4)	11 (12.7)	24 (9.9)	

Continued

Supplemental Table S1. Continued.

Characteristics	Participants follow-up with analysed retina pictures and full data (n = 245)	Participants follow-up with poor quality retina pictures and/or missing data (n = 87)	Non-participants follow-up (n = 242)	Overall p-value
Mother				
Smoking behavior during pregnancy				0.10
Never smoked	167 (68.2)	61 (70.1)	140 (57.8)	
Stopped smoking before pregnancy	46 (18.8)	17 (19.6)	59 (24.4)	
Smoked during pregnancy	32 (13.0)	9 (10.3)	43 (17.8)	
Education level				<0.0001
Low (no high school diploma)	16 (6.5) [a]	6 (6.9) [b]	49 (20.3) [a,b]	
Middle (high school diploma)	64 (26.1)	33 (37.9)	109 (45.0)	
High (college degree or higher)	165 (67.4)	48 (55.2)	84 (34.7)	
Child				
Birth weight, g	3446.6 (429.8)	3447.1 (459.6)	3446.2 (418.4)	0.99
Birth length, cm	50.4 (1.9)	50.6 (1.8)	50.4 (1.9)	0.76

Continued

Supplemental Table S1. Continued.

Characteristics	Participants follow-up with analysed retina pictures and full data (n = 245)	Participants follow-up with poor quality retina pictures and/or missing data (n = 87)	Non-participants follow-up (n = 242)	Overall p-value
Child				
Ethnicity				<0.0001
European	230 (93.9) [a]	82 (94.3) [b]	197 (81.4) [a,b]	
Non-European	15 (6.1)	5 (5.7)	45 (18.6)	
Sex				0.54
Male	116 (47.3)	47 (54.0)	122 (50.4)	
Female	129 (52.7)	40 (46.0)	120 (49.6)	
Exposure				
$PM_{2.5}$, µg/m³				
Pregnancy				
Trimester 1	14.3 (5.5)	13.0 (4.9)	13.6 (5.0)	0.10
Trimester 2	14.3 (5.1)	13.9 (5.2)	13.6 (5.1)	0.29
Trimester 3	14.2 (5.7)	13.9 (5.3)	14.2 (5.3)	0.89
Entire pregnancy	14.3 (2.3) [a,b]	13.6 (2.5) [a]	13.8 (2.4) [b]	0.02

Continued

Supplemental Table S1. Continued.

Characteristics	Participants follow-up with analysed retina pictures and full data (n = 245)	Participants follow-up with poor quality retina pictures and/or missing data (n = 87)	Non-participants follow-up (n = 242)	Overall p-value
$PM_{2.5}$, µg/m³				<0.0001
European	230 (93.9) [a]	82 (94.3) [b]	197 (81.4) [a,b]	
Childhood				
Average childhood exposure	12.6 (1.1)	12.6 (1.2)	12.5 (1.3)	0.54
NO_2, µg/m³				
Pregnancy				
Trimester 1	14.3 (5.5)	13.0 (4.9)	13.6 (5.0)	0.10
Trimester 2	14.3 (5.1)	13.9 (5.2)	13.6 (5.1)	0.29
Trimester 3	14.2 (5.7)	13.9 (5.3)	14.2 (5.3)	0.89
Entire pregnancy	14.3 (2.3) [a,b]	13.6 (2.5) [a]	13.8 (2.4) [b]	0.02
Childhood				
Average childhood exposure	17.2 (3.4)	17.1 (3.3)	17.5 (3.8)	0.58

Groups with significant differences are indicated with the letters 'a' and 'b'. Abbreviations: BMI, body mass index; SD, standard deviation.

<u>Supplemental Table S2.</u> Associations between either CRAE or CRVE and NO_2 exposure during pregnancy, stratified for mean arterial pressure.

	Window of NO_2 exposure	CRAE			CRVE		
		Change, µm	95% CI	p-value	Change, µm	95% CI	p-value
Low MAP	Entire pregnancy	2.25	-1.81 to 8.65	0.27	3.41	-1.84 to 8.65	0.20
High MAP	Entire pregnancy	3.87	0.04 to 7.69	0.05	4.71	-0.72 to 10.13	0.09

The cut-off value for stratification of the MAP in a low and high group was determined by the median value of the study population, equaling 68.22 mmHg. Estimates are given as change in CRAE or CRVE and the according 95% confidence interval (CI) for every IQR increase in exposure to NO_2 within the entire period of pregnancy. All models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. Abbreviations: CI, Confidence interval; CRAE, Central retinal venular equivalent; CRVE, Central retinal venular equivalent; MAP, mean arterial pressure; NO_2, Nitrogen dioxide.

Supplemental Table S3. Sensitivity analysis excluding mothers with hypertension (n = 8) and gestational diabetes (n = 15).

		CRAE		CRVE		TI	
	Window of exposure	**Change, µm**	**95% CI**	**Change, µm**	**95% CI**	**Change**	**95% CI**
$PM_{2.5}$	Trimester 1	2.23	-2.73 to 7.19	1.21	-5.40 to 7.82	0.0014	-0.0029 to 0.0057
	Trimester 2	1.47	-1.75 to 4.70	3.00	-1.30 to 7.30	0.00003	-0.0028 to 0.0028
	Trimester 3	3.69	-1.19 to 8.58	2.65	-3.86 to 9.15	0.0032	-0.0011 to 0.0074
	Entire pregnancy	2.71	-0.14 to 5.57	3.41	-0.40 to 7.21	0.0014	-0.0011 to 0.0039
NO_2	Trimester 1	0.51	-3.02 to 4.05	1.48	-3.22 to 6.17	0.0014	-0.0017 to 0.0043
	Trimester 2	1.43	-2.48 to 5.35	2.09	-3.11 to 7.29	-0.0006	-0.0039 to 0.0028
	Trimester 3	1.42	-2.26 to 5.10	1.61	-3.27 to 6.50	0.0037*	0.0006 to 0.0069
	Entire pregnancy	2.54	-0.18 to 5.27	3.68*	0.06 to 7.31	0.0027*	0.0004 to 0.0051

Estimates are given as change in CRAE, CRVE or TI and the according 95% confidence interval (CI) for every IQR increase in exposure to either $PM_{2.5}$ or NO_2 within the three trimesters and entire period of pregnancy. All models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. Models investigating trimester-specific exposure were adjusted for the three pregnancy trimester-averaged exposures levels. Abbreviations: CI, Confidence interval; CRAE, Central retinal venular equivalent; CRVE, Central retinal venular equivalent; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; TI, Tortuosity index. * ($p \leq 0.05$).

Supplemental Table S4. Sensitivity analysis excluding participants born from premature pregnancies (gestational age lower than 37 weeks) (n = 12).

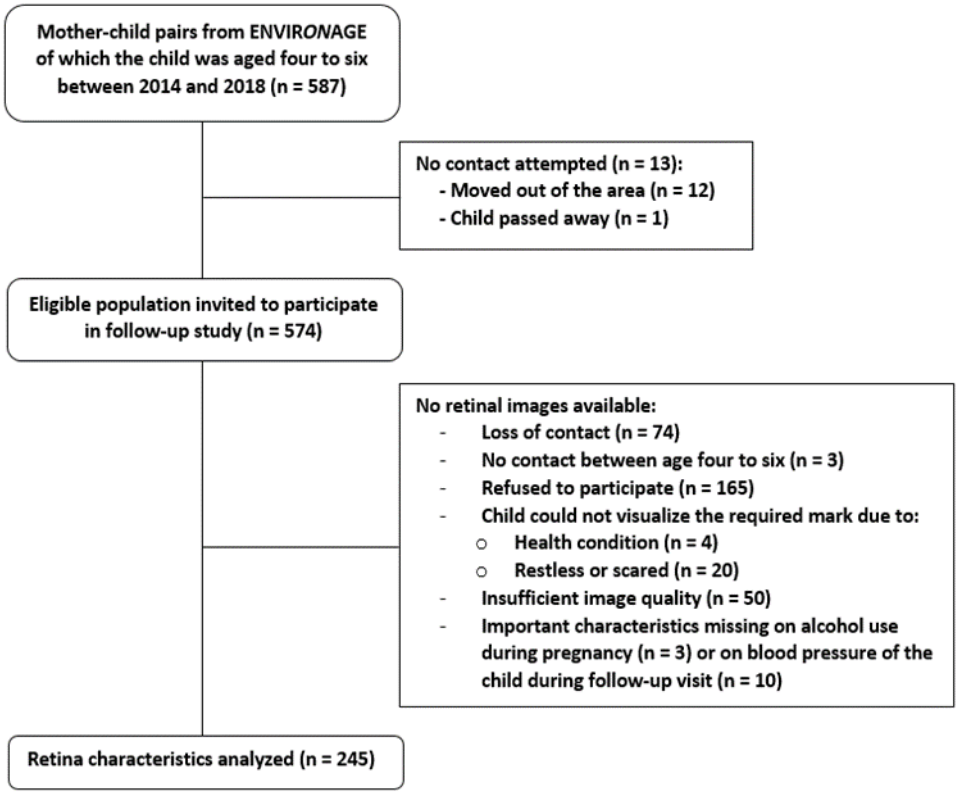
		CRAE		CRVE		TI	
	Window of exposure	**Change, µm**	**95% CI**	**Change, µm**	**95% CI**	**Change**	**95% CI**
$PM_{2.5}$	Trimester 1	1.67	-3.12 to 6.45	2.28	-4.22 to 8.78	0.0035	-0.0006 to 0.0076
	Trimester 2	1.67	-1.55 to 4.90	2.81	-1.57 to 7.19	0.0002	-0.0026 to 0.0030
	Trimester 3	3.17	-1.69 to 8.02	3.55	-3.04 to 10.14	0.0046*	0.0004 to 0.0088
	Entire pregnancy	2.58	-0.29 to 5.46	3.56	-0.34 to 7.74	0.0021	-0.0004 to 0.0045
NO_2	Trimester 1	0.62	-2.80 to 4.04	2.41	-2.22 to 7.04	0.0026	-0.0003 to 0.0055
	Trimester 2	1.77	-2.15 to 5.69	1.89	-3.41 to 7.20	-0.0011	-0.0045 to 0.0022
	Trimester 3	1.23	-2.40 to 4.86	2.10	-2.80 to 7.01	0.0043*	0.0012 to 0.0073
	Entire pregnancy	2.74*	0.02 to 5.47	4.35*	0.67 to 8.04	0.0033*	0.0010 to 0.0056

Estimates are given as change in CRAE, CRVE or TI and the according 95% confidence interval (CI) for every IQR increase in exposure to either $PM_{2.5}$ or NO_2 within the three trimesters and entire period of pregnancy. All models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. Models investigating trimester-specific exposure were adjusted for the three pregnancy trimester-averaged exposures levels. Abbreviations: CI, Confidence interval; CRAE, Central retinal venular equivalent; CRVE, Central retinal venular equivalent; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; TI, Tortuosity index. * ($p \leq 0.05$).

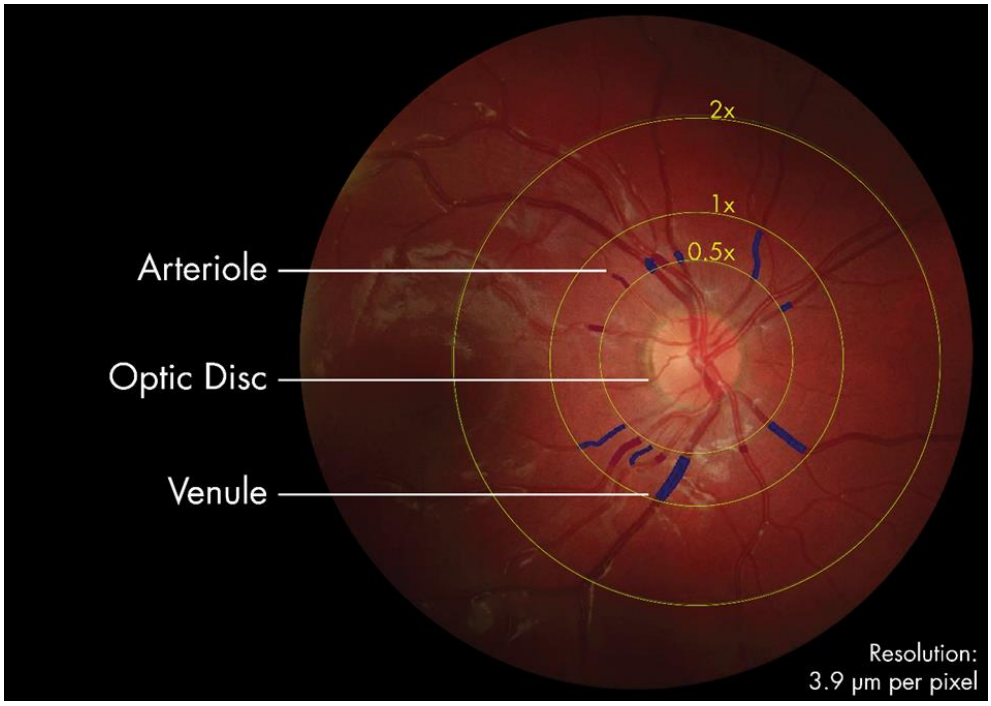
Supplemental Table S5. Sensitivity analysis excluding participants who smoked during pregnancy (n = 32).

		CRAE		CRVE		TI	
	Window of exposure	**Change, µm**	**95% CI**	**Change, µm**	**95% CI**	**Change**	**95% CI**
$PM_{2.5}$	Trimester 1	3.02	-1.95 to 7.98	4.60	-2.25 to 11.45	0.0028	-0.0016 to 0.0072
	Trimester 2	1.87	-1.52 to 5.26	2.55	-2.13 to 7.22	0.0002	-0.0028 to 0.0032
	Trimester 3	3.64	-1.23 to 8.52	4.04	-2.68 to 10.77	0.0033	-0.0010 to 0.0077
	Entire pregnancy	3.10*	0.13 to 6.08	3.92	-0.18 to 8.02	0.0018	-0.0008 to 0.0045
NO_2	Trimester 1	1.70	-1.97 to 5.38	4.41	-0.63 to 9.45	0.0029	-0.0003 to 0.0062
	Trimester 2	1.04	-3.08 to 5.17	0.02	-5.65 to 5.96	-0.0013	-0.0049 to 0.0023
	Trimester 3	1.32	-2.42 to 5.06	2.11	-3.02 to 7.25	0.0036*	0.0003 to 0.0069
	Entire pregnancy	2.79*	-0.002 to 5.58	3.85*	0.002 to 7.69	0.0030*	0.0005 to 0.0054

Estimates are given as change in CRAE, CRVE or TI and the according 95% confidence interval (CI) for every IQR increase in exposure to either $PM_{2.5}$ or NO_2 within the three trimesters and entire period of pregnancy. All models were adjusted for age (years), sex, ethnicity, mean arterial blood pressure and BMI of the child at the moment of the follow-up visit, the season in which the follow-up examination took place, birth weight (grams), maternal age at the birth of her child and pre-pregnancy BMI, maternal education level, alcohol use of the mother during pregnancy, smoking habits of the mother before and during pregnancy, and the exposure of the child to passive smoking. Models investigating trimester-specific exposure were adjusted for the three pregnancy trimester-averaged exposures levels. Abbreviations: CI, Confidence interval; CRAE, Central retinal venular equivalent; CRVE, Central retinal venular equivalent; NO_2, Nitrogen dioxide; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; TI, Tortuosity index. * ($p \leq 0.05$).



Supplemental Figure S1. Flow chart of the selection process resulting in the 245 participants of this study.



Supplemental Figure S2. The central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE) were calculated within 0.5 and 1 times the diameter of the optic disc, starting from its margin. Arterioles within this area are indicated in red, the venules in blue.

CHAPTER 5

ASSOCIATION OF RETINAL MICROVASCULAR CHARACTERISTICS WITH SHORT-TERM MEMORY PERFORMANCE IN CHILDREN AGED 4 TO 5 YEARS

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ABSTRACT

Importance: Neurocognitive functions develop rapidly in early childhood and depend on the intrinsic cooperation between cerebral structures and the circulatory system. The retinal microvasculature can be regarded as a mirror image of the cerebrovascular circulation.

Objective: To investigate the association between retinal vessel characteristics and neurological functioning in children aged 4 to 5 years.

Design, setting, and participants: In this cohort study, mother-child pairs were recruited at birth from February 10th 2010, to June 24th 2014, and renewed consent at their follow-up visit from December 10th 2014, to July 13th 2018. Participants were followed up longitudinally within the prospective Environmental Influence on Aging in Early Life birth cohort. A total of 251 children underwent assessment for this study. Data were analyzed from July 17th to October 30st 2019.

Main outcomes and measures: Retinal vascular diameters, the central retinal arteriolar equivalent (CRAE), central retinal venular equivalent (CRVE), vessel tortuosity, and fractal dimensions were determined. Attention and psychomotor speed, visuospatial working memory, and short-term visual recognition memory were assessed by the Cambridge Neuropsychological Test Automated Battery, including the following tasks: Motor Screening (MOT), Big/Little Circle (BLC), Spatial Span (SSP), and Delayed Matching to Sample (DMS).

Results: Among the 251 children included in the assessment (135 girls [53.8%]; mean [SD] age, 4.5 [0.4] years), for every 1-SD widening in CRVE, the children performed relatively 2.74% (95% CI, -0.12 % to 5.49 %; p = 0.06) slower on the MOT test, had 1.76% (95% CI, -3.53% to -0.04%; p = 0.04) fewer correct DMS assessments in total, and made 2.94% (95% CI, 0.39% to 5.29%; p = 0.02) more errors given a previous correct answer in the DMS task on multiple linear regression modeling. For every 1-SD widening in CRAE, the total percentage of errors and errors given previous correct answers in the DMS task increased 1.44% (95% CI, -3.25% to 0.29%; p = 0.09) and 2.30% (95% CI, -0.14% to 4.61%; p = 0.07), respectively. A 1-SD higher vessel tortuosity showed a

4.32% relative increase in latency in DMS task performance (95% CI, -0.48% to 9.12%; p = 0.07). Retinal vessel characteristics were not associated with BLC and SSP test outcomes.

Conclusions and relevance: These findings suggest that children's microvascular phenotypes are associated with short-term memory and that changes in the retinal microvasculature may reflect neurological development during early childhood.

INTRODUCTION

Neurological development is at its most crucial phase during early childhood. From early gestation, important brain structures are formed and re-shaped, and dendritic connections of neuronal networks are constantly established until about 5 years of age.[214] Cognitive performance depends on a proper functioning cerebral blood circulation since this part of the vascular network entails approximately 15% of the total human cardiovascular output.[215] Various studies have shown that failure to maintain a good cardiovascular health from a young age onwards results in a lower scoring of numerous neurological outcomes later in life, such as psychomotor speed, executive function, and verbal memory.[216,217]

The microcirculation is a large part of the human vasculature that undergoes extensive, organ-specific perinatal maturation.[187,188] The retina is an outgrowth of the developing brain and both tissues share morphological and functional characteristics. Therefore, the retinal microvasculature can be considered as a proxy of the conditions of the blood vessels in the brain.[218] Structural changes in the retinal vasculature can be early markers for the development of cerebral vascular disease.[219,220] In previous research, smaller retinal arteriolar calibres, summarized as the central retinal arteriolar equivalent (CRAE), have been linked to a lower IQ score children aged 11 years.[199] However, to date, retinal vessel diameters and other metrics, such as tortuosity, have never been studied in association with neurocognitive functioning in children as young as 4 years.

We have studied the association between retinal microvascular characteristics and neurocognitive functioning in children aged 4 to 5 years in the prospective ENVIRonmental influence *ON* AGEing in early life (ENVIR*ON*AGE) birth cohort. By means of a *Cambridge Neuropsychological Test Automated Battery* (CANTAB) test battery, we investigated the association between the retinal vasculature and psychomotor speed, visuospatial short-term working memory, and visual short-term recognition memory.

MATERIALS AND METHODS

Study population

Mother-child pairs participating in this study were recruited as part of the ongoing prospective ENVIR*ON*AGE birth cohort. Study protocols of the recruitment phase at birth (from February 10th 2010, to June 24th 2014) and the follow-up phase at the age of 4 were approved by the ethics committees of both the Hospital East-Limburg, Genk, Belgium and Hasselt University, Diepenbeek, Belgium. Details on recruitment of eligible mother-child pairs is described elsewhere.[88] Mothers and their children were re-invited to participate in the first follow-up visit of this prospective cohort study when the child was aged 4 to 6 years. Written consent was obtained from all participating mothers, and children gave their oral assent. Follow-up examinations took place from December 10th 2014 to July 13th 2018. Within this timeframe, 587 children were aged 4 to 6 and could participate in this phase of the study. Thirteen mother-child pairs were not eligible for participation since their child had died (n = 1) or they had moved abroad or too far from our examination center (n = 12). Of the remaining 574 mother-child pairs, 74 women could not be contacted since no up-to-date contact details could be retrieved, 3 mother-child pairs could not be contacted within the timeframe that the child was 4 to 6 years of age, 165 women refused to participate, and 332 renewed consent. Hence, this resulted in a final participation rate of 57.8%. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Of the 332 participating mother-child pairs, 74 were not included in the statistical analyses since no good-quality images of the retinal vasculature of either eye were available. Pictures could not be obtained in 24 participants owing to a medical condition (n = 4) or a lack of concentration or unwillingness to participate (n = 20). For 50 participants, the quality of the pictures was suboptimal, owing to children's movement of their body or eyes during the capturing of the images. Finally, for 7 additional participants, no CANTAB data could be obtained, resulting in a study population of 251 (**Supplemental Figure S1**).

At the follow-up visit, the mothers filled out several questionnaires to obtain general information on the lifestyle of the child and the parents, the emotional

wellbeing of the mother at the moment of the follow-up examinations, and on the emotions and behaviour of their child, summarized during the last 6 months before the follow-up examination via the Strengths and Difficulties questionnaire (SDQ), which has been validated in other cohorts studying children in this age group.[221] For the Strengths and Difficulties Questionnaire, the first 25 of a total of 33 questions are subdivided into 5 subscales: Hyperactivity, Emotional Symptoms, Conduct Problems, Peer Problems, and Prosocial Behavior. The scores of the first 4 subscales were summarized into a problem score, with a final score ranging from 0 to 40 and higher scores indicating a more problematic emotional behavior.

Clinical measurements

A single trained examiner performed the measurements of the clinical parameters (height, weight and blood pressure) (N.M.), took the fundus pictures (L. J. L.) and gave the instructions for the administration of the CANTAB test battery (L. J. L.). It was made clear at the beginning of the clinical examinations that each test could be stopped at any moment if the child was feeling uncomfortable or scared. The participant's blood pressure was measured with an automated upper-arm blood-pressure monitor (Omron 705IT, Omron Corporation, Japan), equipped with a cuff adapted to the arm size of children. Measurements were performed by a standardized method, as described by the European Society of Hypertension.[195] In summary, after five minutes of rest in supine position, a trained observer (N.M.) obtained five consecutive readings of the systolic (SBP) and diastolic (DBP) blood pressure with 1-minute intervals. Average systolic and diastolic blood pressure were based on the mean of the last three readings. Mean arterial pressure (MAP) was calculated via the following equation: MAP = (2/3 * DBP) + (1/3 * SBP).

To determine the retinal blood vessel parameters, fundus pictures of the right and left eyes were obtained with a 45° 6.3 megapixel digital nonmydriatic retinal camera (Canon CR-2 plus, Hospithera, Brussels, Belgium). These pictures were subsequently analyzed with the MONA-REVA® software (version 2.1.1, VITO Health, Mol, Belgium). First, the diameter of the optic disc (OD) was determined for each picture and all distance measurements within the fundus were set relative to this value. Next, the widths of the retinal arterioles and venules were calculated

in the zone between 0.5 and 1 times the diameter of the OD. The diameters of the 6 largest arterioles and 6 largest venules in this zone were used in the revised Parr-Hubbard formula to calculate the central retinal arteriolar equivalent (CRAE) and central retinal venular equivalent (CRVE).[197] The tortuosity index (TI) is computed as the mean tortuosity of the branch segments, where the tortuosity of a branch segment is the ratio of the line traced on each tree along the vessel axis between 0.5 and 2 times the OD diameter and the line connecting the endpoints. Segmentations are cropped centered on the OD whereby the inner and outer radii were taken at 1.5 and 5.0 times the radius of the OD. [198] Finally, multifractal analysis was performed in the FracLac plugin in ImageJ (Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA) using the segmentations computed by MONA-REVA. The applied segmentation algorithm used multiscale line filtering based on Nguyen *et al.* [198] and post-processing steps such as double thresholding, blob extraction, removal of small connected regions and filling holes. Settings of the ImageJ fractal dimension extraction in the FracLac plugin were: box-counting method, number of grid positions: 10, calculating grid calibers: scaled series, and Q range = [-10; +10; 0.1]. We calculated the generalized dimensions (Dq) for q = 0, 1 and 2, which are respectively the capacity dimension (D0), the information dimension (D1) and correlation dimension (D2), the curve asymmetry, and the singularity length Δα. For a multifractal structure the following applies: $D0 \geq D1 \geq D2$.[222]

When both fundus images of the child were available (n=201), the mean values of the CRAE, CRVE, and TI were used in the analyses. For 29 individuals, the calculations were available for the left eye only, whereas for 21 children, the data of the right eye only were used for analysis. For these 50 individuals, the fundus image of the other eye was unavailable or of insufficient quality. No difference could be determined between the CRAE ($p = 0.54$), CRVE ($p = 0.39$) or TI ($p = 0.31$) of either 1 or 2 eyes.

CANTAB test battery

The neurocognitive development of the children was assessed by means of 4 tasks (**Figure 1**) of the *Cambridge Neuropsychological Test Automated Battery* (CANTAB; Cambridge, United Kingdom), performed on a touch screen tablet. All

instructions necessary to understand and complete the tasks were given according to a standardized script provided by the software developers. The examiner always made sure that the child used only the index finger of their dominant hand. If the child was unsure about whether it was performing the test correctly or if the child did not understand the task, the task description was repeated once during the test. Participants were encouraged when they were completing the test correctly.

The first task of the CANTAB test battery we used was the Motor Screening Task (MOT), which familiarized the child with the touch screen and verified if oral descriptions given by the examiner were well understood. By touching a series of crosses on the screen as fast as possible, the MOT assesses the reaction speed (latency in milliseconds) and the mean error, which measures the accuracy of touch in pixel units between the point of touch and the center of the target (based on a screen resolution of 640 by 350 pixels).

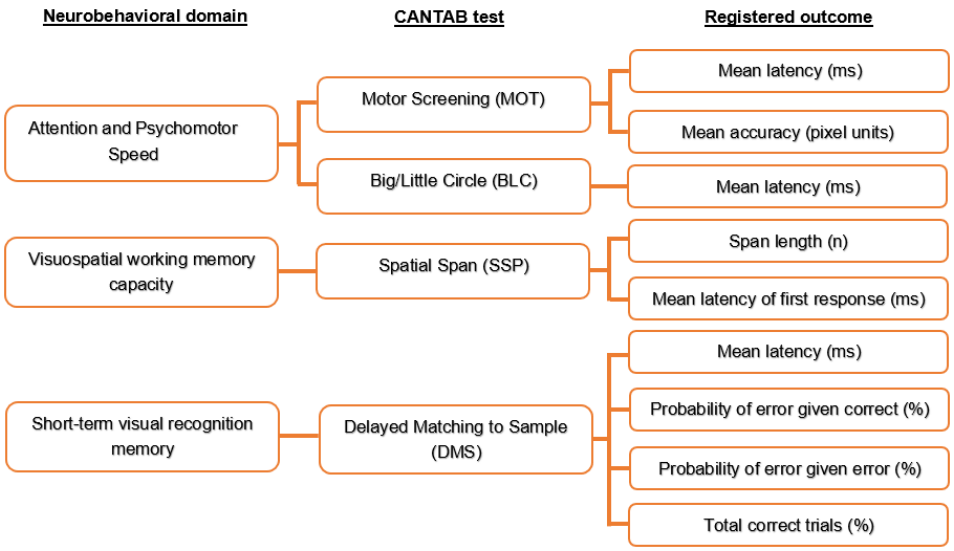


Figure 1. Schematic representation of the four CANTAB tests used in this study, with the corresponding neurobehavioral domains that are assessed with these tests, and the specific outcomes that are registered for each of the tasks.

Next, the child executes the Big/Little Circle (BLC) task. Similar to the MOT, this test familiarizes the participant with oral task descriptions and a touch screen. The child first had to select the smallest of two circles. After a series of 20 assessments, the child now had to touch the biggest circle for another series of 20 tests. The outcome measures of the BLC task covers the speed of response (latency in milliseconds), as well as the number of correct answers. Both the MOT and BLC task perform an assessment of attention and psychomotor speed.

Subsequently, the child performed the Spatial Span (SSP) task to assess short-term memory. Nine white squares were shown on the screen, of which initially 2 boxes changed color in a certain sequence. The child then had to touch the boxes in the same order as they changed color. When the task was performed correctly, an additional box changed color in the next sequence, for up to a series of 9 boxes. The sequences varied throughout the test. The child could try again if he or she choose the wrong sequence or square, for a maximum of 3 times. After 3 subsequent errors, the test was ended. Outcome measures for the SSP task are given as the maximum number of boxes that the child could remember in the correct sequence (span length), and the time to initiate touching the sequence (mean latency to first answer in milliseconds). Finally, the Delayed Matching to Sample (DMS) task was performed, which measures visual matching ability and visual recognition memory. In the center of the screen, a complex visual pattern was shown. After several seconds, the child had to choose this pattern from 4 similar patterns. These choice figures appeared on the screen either simultaneously with the first (sample) figure, or with a delay of either 4 or 12 seconds. Reported outcomes are the percentage of correct answers (all assignments or only those at which the choice patterns are shown with a delay) and the latency from when the choice patterns were displayed until choosing the correct pattern, if the first touched choice was the correct one.

Statistics

Data were analyzed from July 17th to October 30st, 2019. We used SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA) for data analyses. Correlations between the retinal vessel characteristics were assessed by Pearson correlation.

We tested differences between the retinal arteriolar and venular diameter, retinal vessel curvature of either one or two eyes, and of microvascular and CANTAB outcomes between boys and girls with a 2-sided *t* test. The latency data of MOT, BLC, SSP and DMS were log-transformed to better comply with linear model assumptions. Other test outcomes were studied without additional transformation. We combined CANTAB test outcomes assessing the same neurological area into 2 different principal components (PC) using the PROC PINCOMP procedure. We then used separate multivariable linear regression models to estimate associations between retinal vessel characteristics and cognitive functioning, using the first 2 principal components as the dependent variables. These first 2 principal components combined, explained more than 50% of the data variance. Subsequently, we tested the associations between retinal vessel characteristics and all separate outcome variables of the 4 CANTAB tasks in the battery, by means of multivariable linear regression models. All models were adjusted for the child's sex, age, body mass index (BMI; calculated as weight in kilograms divided by height in meters squared), gestational age, birth weight and ethnicity. In a subsequent analysis of the models assessing the separate test outcomes, we additionally adjusted for the MAP, the season in which the follow-up examination took place, the time of day at which the CANTAB task was performed, and the problem score, as a representation of emotional state and behavior. The magnitude of the association was expressed for every 1-SD increment in retinal vessel characteristic.

We performed 4 additional sensitivity analyses. In the first sensitivity analysis we additionally adjusted the model for the education level of both mother and father at the moment of the follow-up examination, the smoking habits and alcohol use of the mother during pregnancy, and the exposure of the child to passive smoking of the parents. Secondly, a sensitivity analysis was conducted to assess the association between retinal vessel characteristics and CANTAB task outcomes in a population excluding mothers who consumed alcohol during pregnancy (n = 50), since prenatal exposure to alcohol is known to have substantial effects on neurological development of the fetus, which can also be reflected by changes in retinal vessel tortuosity and various other biomarkers.[223] A third sensitivity analysis was performed in the subset of children excluding those with a high

problem score assessed with the Strengths and Difficulties Questionnaire (i.e. between 14 and 40 points) (n = 33). A final sensitivity analysis was conducted, excluding children who were born prematurely (i.e. ≤ 37 weeks of gestation) (n = 11).

RESULTS

Study population characteristics

The study characteristics of the 251 participating mother-child pairs are provided in **Table 1**. On the day of the measurements, the children (135 girls [53.8%] and 116 boys [46.2%]) had a mean [SD] age of 4.5 [0.4] years and MAP of 68.6 [6.0] mm Hg. Mean BMI was 16.0 [1.5] kg/m². Examinations mostly took place during spring (82 [32.7%]) and winter (77 [30.7%]). In their home environment, 39 children (15.5%) were exposed to passive smoking via 1 parent and 34 (13.5%) via both parents. Mothers gave birth at a mean [SD] age of 29.9 [4.2] years. Most of the mothers did not consume alcohol (196 [78.1%]) and had never smoked (174 [69.3%]) throughout their pregnancy. Most mothers (178 [70.9%]) had a college education or higher, as did fewer fathers (118 [47.0%]) (**Table 1**).

Microvasculature characteristics

For the entire study population, the mean [SD] measurement of the retinal arteriolar and venular diameter was 180.9 [14.4] µm and 251.1 [19.6] µm, respectively, and the mean [SD] vessel tortuosity value was 0.889 [0.012] (**Table 2**). These 3 retinal vessel characteristics were slightly increased in girls compared with boys, with a small but statistically significant difference for the CRVE (253.4 [18.1] vs 248.3 [20.9] µm; P = 0.04). The multifractal outcomes of the study population were similar to those of the boys and girls separately, with 1.56 [0.03] for D0 (capacity dimension), 1.52 [0.03] for D1 (information dimension), 1.51 [0.03] for D2 (correlation dimension), 0.27 [0.05] for curve asymmetry, and a singularity length of 0.76 [0.07] for all children combined. A positive correlation was found between both the arteriolar and venular diameter (r = 0.58; P < 0.001) and the venular diameter and vessel tortuosity (r = 0.18; P = 0.004), and a borderline positive correlation was found between retinal arteriolar diameter and vessel tortuosity (r = 0.12; P = 0.06).

Table 1. Characteristics of the mother-child pairs included in this study (n = 251).

Characteristic	Mean (SD) or number of participants (%)
Child	
Age at time of follow-up, years	4.5 (0.4)
Sex	
Male	116 (46.2)
Female	135 (53.8)
Ethnicity	
European	236 (94.0)
Non-European	15 (6.0)
Height at time of follow-up, cm	107.7 (5.0)
Weight at time of follow-up, kg	18.7 (2.7)
BMI at time of follow-up, kg/m²	16.0 (1.5)
Season at follow-up	
Spring	82 (32.7)
Summer	51 (20.3)
Autumn	41 (16.3)
Winter	77 (30.7)
Systolic blood pressure, mmHg	97.7 (8.2)
Diastolic blood pressure, mmHg	54.0 (6.9)
Mean arterial pressure, mmHg	68.6 (6.0)
Exposure to passive smoking	
Not exposed	166 (66.1)
Exposed via one parent	39 (15.5)
Exposed via both parents	34 (11.2)
Information missing	18 (7.2)

Continued

Table 1. Continued.

Characteristic	Mean (SD) or number of participants (%)
Mother	
Age at birth child, years	29.9 (4.2)
Pre-pregnancy BMI, kg/m²	24.4 (4.7)
Smoking behavior during pregnancy	
Never smoked	174 (69.3)
Stopped smoking before pregnancy	43 (17.1)
Smoked during pregnancy	34 (13.6)
Alcohol consumption during pregnancy	
Yes	50 (19.9)
No	196 (78.1)
Information missing	5 (2.0)
Education level	
Low (no high school diploma)	14 (5.6)
Middle (high school diploma)	59 (23.5)
High (college degree or higher)	178 (70.9)
Father	
Education level	
Low (no high school diploma)	16 (6.4)
Middle (high school diploma)	106 (42.2)
High (college degree or higher)	118 (47.0)
Information missing	11 (4.4)

Abbreviations: BMI, Body mass index; SD, standard deviation.

Table 2. Average (SD) CRAE, CRVE, TI and multifractal characteristics of the total study population and stratified by sex.

Characteristic	Combined	Girls	Boys	P-value [a]
CRAE, µm	180.9 (14.4)	182.4 (13.6)	179.3 (15.2)	0.10
CRVE, µm	251.1 (19.6)	253.4 (18.1)	248.3 (20.9)	0.04
TI [b]	0.889 (0.012)	0.890 (0.012)	0.887 (0.013)	0.07
D_0	1.56 (0.03)	1.56 (0.03)	1.56 (0.03)	0.63
D_1	1.52 (0.03)	1.52 (0.03)	1.52 (0.04)	0.65
D_2	1.51 (0.03)	1.51 (0.03)	1.50 (0.04)	0.66
Curve asymmetry	0.27 (0.05)	0.27 (0.05)	0.27 (0.06)	0.69
Singularity length	0.76 (0.07)	0.76 (0.07)	0.76 (0.07)	0.77

Abbreviations: CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; D0, capacity dimension; D1, information dimension; D2, correlation dimension; TI, Tortuosity index.

[a] Calculated using a 2-sided t test.

[b] Computed as the mean tortuosity of the branch segments, where the tortuosity of a branch segment is the ratio of the line traced on each tree along the vessel axis from 0.5 to 2.0 times the optic disc diameter and the line connecting the end points.

Cognitive characteristics

CANTAB task outcomes are summarized in **Table 3**, for all participants and for boys and girls separately. All latency outcomes are expressed as geometric means. There was no significant difference in task performance between boys and girls. The mean time to touch the target of the MOT task was 949.2 (95% CI, 920.7-978.6) milliseconds. Mean accuracy of the MOT task was 13.9 (95% CI, 13.6-14.3) pixel units removed from the center of the target cross. The latency of the BLC task was similar to that of the MOT task: the mean time needed to tap the correct circle was 1075.3 (95% CI, 1050.5-1100.7) milliseconds.

The SSP task length ranged from 0 to 5 correct squares tapped within the same sequence, with a mean span length of 2.7 (95% CI, 2.6-2.9) squares. The mean time needed to initiate the SSP assignments was 3666.3 (95% CI, 3498.2-3842.4) milliseconds. Finally, the mean percentage of trials that was answered correctly in the DMS task was 48.3% (95% CI, 46.5%-50.1%) and 39.7% (95% CI, 37.8%-41.5%) when only the assignments in which choice figures were displayed with a delay were taken into account. The percentage of incorrect answers, given that the previous answer was correct, was 54.7% (95% CI, 52.2%-57.1%), and given that the previous answer was incorrect, 48.4% (95% CI, 45.9%-50.8%). The mean time to touch the stimulus on the screen in all DMS trials where the correct stimulus was selected was 4102.2 (95% CI, 3942.5-4268.5) milliseconds, and for only the assignments with delays, 3841.3 (95% CI, 3657.6-4034.1) milliseconds.

Table 3. Average (95% CI) test outcomes of the four tests of the CANTAB cognitive battery (n = 251).

Test and Outcome(s)	Combined	Girls	Boys	P-value
MOT				
Latency, ms	949.2 (920.7-978.6)	957.4 (919.4-997.0)	939.7 (896.8-984.6)	0.55
Mean accuracy, pixel units	13.9 (13.6-14.3)	13.9 (13.4-14.5)	13.9 (13.5-14.3)	0.88
BLC				
Latency, ms	1075.3 (1050.5-1100.7)	1055.5 (1026.6-1085.2)	1098.9 (1057.0-1142.5)	0.10
SSP				
Span length, n	2.7 (2.6-2.9)	2.7 (2.5-2.9)	2.7 (2.5-3.0)	0.93
Latency to first response, ms	3666.3 (3498.2-3842.4)	3704.9 (3477.7-3947.0)	3621.8 (3374.1-3887.6)	0.64
DMS				
Latency, ms	4102.2 (3942.5-4268.5)	4019.2 (3828.0-4220.0)	4201.0 (3936.2-4483.6)	0.28
Latency of trials with delays, ms	3841.3 (3657.6-4034.1)	3700.0 (3476.6-3938.3)	4012.1 (3712.9-4335.5)	0.10
Percentage correct, %	48.3 (46.5-50.1)	49.3 (46.9-51.7)	47.1 (44.2-49.9)	0.23
Percentage correct of trials with delays, %	39.7 (37.8-41.5)	40.5 (38.0-43.1)	38.7 (35.9-41.5)	0.33
Error given correct, %	54.7 (52.2-57.1)	54.7 (51.1-58.2)	54.7 (51.2-58.2)	0.99
Error given error, %	48.4 (45.9-50.8)	47.0 (43.7-50.2)	50.0 (46.3-53.6)	0.23

Abbreviations: BLC, Big/Little Circle; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span.

Associations between cognitive functioning and the retinal microvasculature

Attention and psychomotor speed

Principal components for attention and psychomotor speed were derived as linear combinations of the 3 outcomes of the MOT and BLC task. The first PC explained 49% of the variance, the second PC an additional 34%. Characteristics of the principal components used in the multivariable linear regression models are shown in **Supplemental Table S1**. After adjustment for sex, gestational age, birth weight, ethnicity, BMI and age, CRVE was negatively associated with the second principal component (P = 0.01). None of the other retinal vessel characteristics was associated with the principal components.

With the multiple linear regression analyses for the 3 separate test outcomes, adjusted for the aforementioned variables a negative association between CRVE and the mean accuracy of the MOT task (-0.39 pixel units; 95% CI: -0.78 to -0.10; p = 0.01) was identified. This was accompanied by a borderline positive association between the CRVE and the reaction time of the MOT test: for every 1-SD increase in CRVE, the children performed 2.74% (95% CI: -0.12 to 5.49; p = 0.06) slower on the MOT test (**Figure 2A**). There was no relation between any of the retinal vessel characteristics and the reaction speeds recorded in the BLC task (**Figure 2A**). Further adjustments for MAP, follow-up season, time of CANTAB testing, and problem score decreased the estimated MOT latency for every 1-SD widening in CRVE (1.76%; 95% CI: -1.37 to 5.10; p = 0.27) but did not substantially alter the other estimates for the MOT and BLC task (**Supplemental Table S2**).

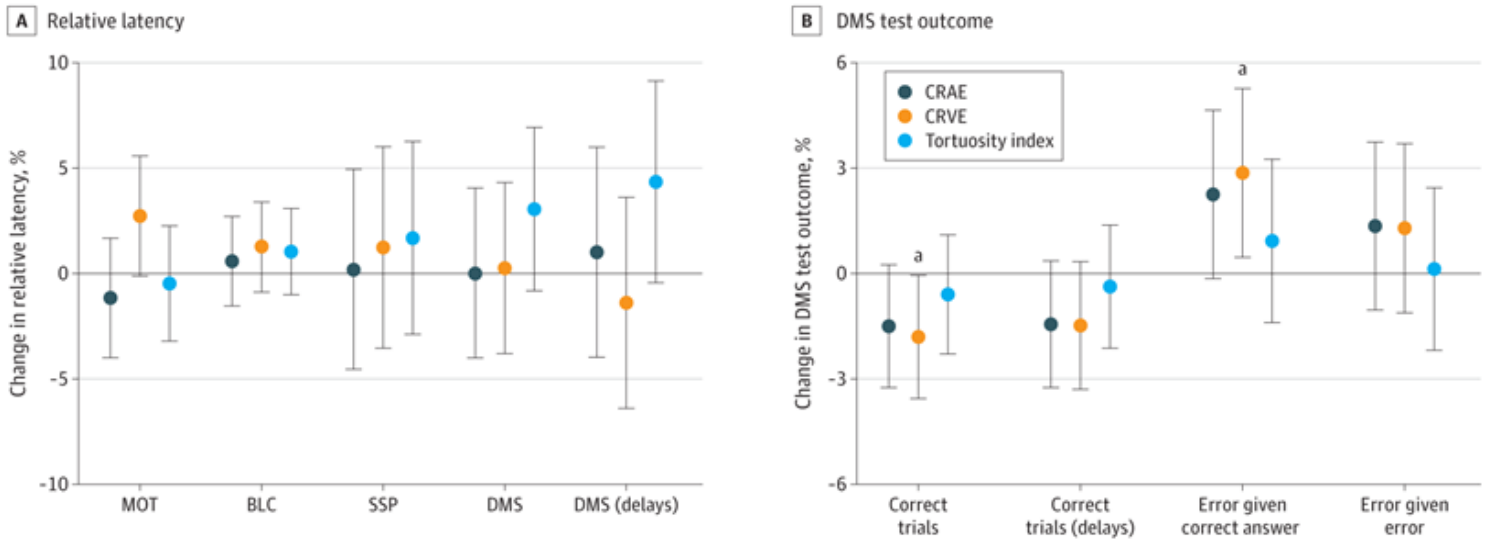


Figure 2. Associations Between the Central Retinal Arterial Equivalent (CRAE) and Central Venular Arteriolar Equivalents (CRVE) and Tortuosity Index and the mean latencies. Latencies were measured for each of the 4 Cambridge Neuropsychological Test Automated Battery tasks (A) and 3 DMS task outcomes (B). The magnitude of all estimates is expressed for every 1-SD increment in retinal vessel characteristic. Multiple linear regression models were adjusted for age, sex, body mass index, and ethnicity of the child. Abbreviations: BLC, Big/Little Circle task; DMS, Delayed Matching to Sample; MOT, Motor Screening task; and SSP, Spatial Span task. Error bars indicate 95% CIs. [a] $P \leq 0.05$.

Short-term memory assessments: visuospatial working memory and visual recognition memory

In the principal component analysis for short-term memory, the first principal component explained 40% of the variance; the second PC, an additional 20% to the explained variance of the model (**Supplemental Table S1**). CRVE was positively associated with the first principal component (p = 0.01) when adjusted for sex, gestational age, birth weight, ethnicity, BMI and age. No other significant differences were found.

In the multiple linear regression analyses of the separate SSP and DMS task outcomes, vessel tortuosity showed a borderline significant association with DMS latency, when only DMS trials with delays were considered: for every unit increase in TI, there was a 4.32% (95% CI: -0.48 to 9.12; p = 0.07) slower performance (**Figure 2A**). Following additional adjustments for MAP, follow-up season, time of CANTAB testing, and problem score, the latter estimate increased: a unit increase in TI was associated with a 6.48% (95% CI: 0.84 to 12.12; p = 0.02) slower performance in DMS trails with delays, and a 4.20% (95% CI: -0.36 to 8.76; p = 0.07) increase in latency for all DMS trails (**Supplemental Table S2**). For the DMS task, other assessed parameters than latency showed significant associations with the retinal vessel characteristics. A widening of both vessel types had an effect on the amount of errors given the previous answer being correct: a 1-SD widening of the retinal arteriolar and venular diameter was associated with a 2.30% (95% CI: -2.01 to 4.61; p = 0.07) and 2.94% (95% CI: 0.39 to 5.29; p = 0.02) increase in the amount of errors, respectively. Moreover, the total percentage of correctly answers trials decreased with 1.44% (95% CI: -3.17 to 0.29; p = 0.09) for every 1-SD increase in CRAE, and with 1.76% (95% CI: -3.53 to -0.04; p = 0.04) for a 1-SD increase in CRVE (**Figure 2B**). Every SD increase in D1 and D2 was associated with a 1.71% (95% CI: -0.15 to 3.57; p = 0.07) and a 1.77% (95% CI: -0.15 to 3.57; p = 0.05) increase in correct answered DMS queries, respectively. Additional adjustments did not alter the associations between the DMS outcomes and the CRVE (**Supplemental Table S2**), but increased the estimates for D1 and D2 to 2.10% (95% CI: 0.06 to 4.11; p = 0.04) and 2.16% (95% CI: 0.21 to 4.11; p = 0.03) correct DMS tasks, respectively.

Sensitivity analyses

We reran our models with additional adjustment for pregnancy-related maternal characteristics, but this did not alter the described associations (**Supplemental Table S3**). When excluding mothers who consumed alcohol during pregnancy, the latency parameters in association with CRVE and TI increased: the MOT latency increased to 4.12% (95% CI: 0.98 to 7.25, p = 0.01) for every 1-SD increase in venular diameter, and DMS latency increased to 5.40% (95% CI: -0.24 to 11.04, p = 0.06) for every 1-SD increase in vessel tortuosity (**Supplemental Table S4**).

The third sensitivity analysis, excluding children with a higher indication of emotional stress at the time of CANTAB test administration, did not alter the relation between CRVE and MOT latency, although the association between TI and DMS latency lost its significance. This analysis slightly increased the estimates of the percentage of errors made given a previous correct answer, and slightly decreased the estimates of the percentage of correct answers of the DMS task, in association with both the retinal arteriolar and venular diameter (**Supplemental Table S5**). Finally, the fourth sensitivity analysis, excluding children who were born premature did not change the associations between retinal vessel parameters and CANTAB test outcomes (**Supplemental Table S6**). Only the association between CRVE and MOT latency became significant (3.14%; 0.39 to 6.08, p = 0.03).

DISCUSSION

The microcirculatory system functions as the exchange surface for oxygen, nutrients, and metabolites between the blood and the parenchymal cells of the body's tissues. The retinal vessel structure gives a noninvasive view on the microvasculature, and because the retina can be considered a "window to the brain," it is used as a proxy to study the cerebral microcirculation. Proper functioning and structure of the microvasculature is essential for neurological function and development. In this context, we studied the associations between several domains of neurological development and phenotypes of the retinal

microvasculature in children aged 4 to 5 years enrolled in the longitudinal ENVIR*ON*AGE birth cohort. The key findings of this study are that (1) a wider CRVE and an increased vessel tortuosity are associated with a lower short-term visual recognition memory performance; (2) a wider CRVE is associated with a slower performance in the MOT test; and (3) an increased information dimension (D1) and correlation dimension (D2) are associated with a better performance in short-term memory assessment. This is the first study, to our knowledge, to find an association between retinal vessel characteristics and different areas of neurological development in children as young as 4 years.

Structural changes in the retinal microvasculature have been assessed as potential biomarkers of neurological conditions in adults. For patients with Parkinson's disease, the morphology of the retinal veins, rather than the diameter, seems to be affected by the disease process.[224] Cheung and colleagues found that persons with Alzheimer disease (mean age of 74.8 years) had narrower retinal venules and an increased vessel tortuosity compared with the matched control population.[225] A large follow-up study in 12 317 participants in which neurological tests were performed 3 times during a period of 20 years indicated that a narrowing of the retinal arteriolar diameter was associated with a decrease in 20-year cognitive change, although these results did not persist in more elaborate adjusted models.[226] In 244 patients with type 1 diabetes, both a narrower CRAE and wider CRVE were associated with lower scoring on a set of tests assessing mental efficiency and executive function and with a poorer performance for verbal memory for a narrower arteriolar diameter.[227]

Although neurological development in children has been evaluated under several clinical conditions and environmental exposures [228], few studies have examined the link between cognitive functioning and retinal vessel characteristics in early life. Recently, Wei and colleagues compared retinal vessel diameters between preterm (n = 93) and term-born (n = 87) children at 11 years of age.[199] They found an association between narrowing of the retinal arterioles and a poorer performance in matrix reasoning (-1.77 points; P = 0.004) and spatial span length (-2.03 points; P = 0.002) of the Wechsler Non–Verbal Scale of Ability, the latter in contrast to our findings. A lack of association between SSP test outcomes and

retinal vessel measurements in our study can be explained by the age difference at which the tests were administered: even in infants, 5 months compared with 12 months of age can result in a greater number of correct responses in a simplified form of working memory testing.[229] Moreover, Luciana and Nelson performed several of the CANTAB tasks in children ranging from ages 4 to 12 years and found significant differences in the performance between these age groups.[230] Therefore, it is crucial that our CANTAB tasks are repeated in other cohorts and during the next follow-up steps in our ENVIR*ON*AGE prospective birth cohort.

We found a slower reaction speed in the MOT test in association with a widening of the CRVE. The effect of vessel properties on psychomotor speed have mostly been researched in adults and elderly populations. In a study on 77 adults suffering DMT1, a reduced blood flow in the basal ganglia was associated with slower psychomotor speed.[231] Kim and colleagues looked at a combined measure of microvascular abnormalities and found an association between a high level of microvascular burden and reduced scoring on psychomotor assessment.[232]

Visual short-term memory is a complex neurological phenomenon, regulated by an intrinsic cooperation of different areas of the brain. As early as six months old this memory system becomes apparent and it rapidly develops throughout early childhood.[233,234] A recent follow-up study in 124 children, assessed at 4 and a second time at 7 years of age, revealed that outcomes on visual short-term memory in four-year-olds were predictive of the mathematical achievement at age seven ($r = 0.34$, $p \leq 0.01$).[235] Our results suggest that retinal vessel diameter and tortuosity are an underlying phenomenon relevant in explaining short-term memory, elaborating the current knowledge on the complex process of memory formation and cognitive development.

Neurocognitive processes depend on a proper microvascular architecture, with its functioning intertwined with several factors, such as the endothelial cell activity of the blood-brain barrier.[236,237] Arteriolar and venular diameters determine adequate exchange of oxygen and nutrients to the peripheral tissues, and these metrics are instrumental for the characterization of the microvasculature. Widening of the arterioles and venules could lower the pressure in these vessels,

whereas enough pressure is required for adequate diffusion of oxygen and removal of metabolic waste products.[238] Reduced supply of oxygen and nutrients has been proven an important cause of poorer results in neurocognitive testing.[239,240] These observations, combined with our findings, can provide further insight in a pathophysiological model on the effects of microvascular changes on neurocognitive development, initiated during childhood.

Another potential association between retinal vessel changes and cognition could be increased inflammation. Increased levels of inflammatory markers have been associated with changes in retinal vessel diameter [241] and a decline in cognitive functioning in adults.[242] A wider retinal venular diameter has been associated with higher levels of C-reactive protein, a marker of inflammation, among children aged 6 years in the Generation R Study [243] as well as in a German population of school-aged children (mean [SD] age, 11.1 [0.6] years).[244] Studies in adults have shown that retinopathy [245] and retinal angular and branching abnormalities [246] are associated with decreased neurocognitive scoring. Hypothesized is that these abnormalities give rise to increased shear stress in the microvasculature, resulting in increased inflammatory reactions at the endothelial lining of the blood-brain barrier. Future studies should therefore focus on the potential intermediary role of inflammation in the retina-brain connection.

A strength of this study is that the retinal image analyses were performed by a single researcher who was blinded for all study conditions, thereby excluding examiner bias. Furthermore, not many normative data of CANTAB tasks are available among children as young as 4 years.[247] Only Luciana and Nelson tested a large sample of children at this age.[230,248] With this study, we provide additional normative data on CANTAB test outcomes in a population of healthy children aged 4 to 6 years. A limitation of this study is that performing neurocognitive assessments, such as the CANTAB tasks, in children aged 4 years remains a challenge owing to several age-related factors.[248] For example, owing to insufficient finger pressure or surface area, some children fail to generate a response at first attempt. However, by implying a test round with several trials before commencing each of the 4 tasks, the children learned how much pressure is needed to register their answer, therefore reducing the chance of not generating

enough finger pressure in the registered trials. Moreover, the 4 tasks in the CANTAB battery were determined a priori, taking into consideration the young age of the participants, and by using a standardized script for test administration the proper execution of the tests is directed.

CONCLUSION

In this cohort study, a widening of the retinal venular diameter and increased information dimension (D1) and correlation dimension (D2) were associated with the outcome of established neurodevelopmental tests and can be regarded as potential biomarkers to evaluate neurobehavioral development in children as young as 4 years. These noninvasive retinal measurements can support investigations into microvascular changes that accompany neurological development at a young age. Neurological assessments and retinal imaging at future follow-up visits of the ENVIR*ON*AGE prospective birth cohort will further elaborate these findings.

SUPPLEMENTAL MATERIAL

Supplemental Table S1. Characteristics of the first and second principal components.

Neurocognitive domain	Eigenvalue PC1 [Explained variance]	Eigenvalue PC2 [Explained variance]	CANTAB test outcome	Eigenvector PC1	Eigenvector PC2
Attention and psychomotor speed	1.47 [49%]	1.00 [34%]	MOT latency	0.71	0.06
			MOT mean error	0.06	0.99
			BLC latency	0.70	-0.14
Short term memory	2.40 [40%]	1.20 [20%]	SSP span	-0.24	-0.39
			SSP latency first response	0.09	0.68
			DMS latency	-0.09	0.60
			DMS percentage correct	-0.63	0.07
			DMS error given correct	0.50	-0.14
			DMS error given error	0.53	0.03

Abbreviations: BLC, Big/Little Circle; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span.

Supplemental Table S2. Associations of the multiple linear regression analyses between retinal vessel characteristics and outcomes of the four CANTAB tests, additionally adjusted for MAP, season of follow-up, the time of day at which the CANTAB test was performed, and problem score.

CANTAB test and outcome(s)	CRAE	CRVE	TI
MOT			
Mean latency	-1.01 (-4.32; 2.30)	1.76 (-1.37; 5.10)	-0.84 (-3.96; 2.28)
Mean accuracy	-0.14 (-0.58; 0.14)	-0.78 (-1.18; -0.39)*	0.12 (-0.24; 0.60)
BLC			
Mean latency	1.15 (-1.44; 3.74)	1.37 (-1.18; 3.92)	1.44 (-0.96; 3.84)
SSP			
Span length	-0.06 (-0.29; 0.12)	-0.08 (-0.20; 0.10)	-0.02 (-0.24; 0.12)
Mean latency of first response	1.73 (-4.03; 7.49)	1.76 (-3.92; 7.25)	3.24 (-2.04; 8.64)

Continued

Supplemental Table S2. Continued

CANTAB test and outcome(s)	CRAE	CRVE	TI
DMS			
Mean latency	1.73 (-3.17; 6.62)	1.57 (-3.33; 6.27)	4.20 (-0.36; 8.76)
Mean latency (trials with delays)	2.45 (-3.60; 8.50)	-0.39 (-6.27; 5.49)	6.48 (0.84; 12.12)*
Error given correct	1.44 (-1.30; 4.32)	2.74 (-0.08; 5.29)	0.36 (-2.28; 3.00)
Error given error	1.58 (-1.30; 4.32)	1.76 (-0.98; 4.51)	0.24 (-2.40; 2.88)
Percentage Correct	-1.44 (-3.46; 0.72)	-1.76 (-3.92; 0.18)	-0.24 (-2.16; 1.80)
Percentage Correct (trials with delays)	-1.58 (-3.74; 0.58)	-1.57 (-3.72; 0.59)	-0.24 (-2.28; 1.80)

Estimated changes (% change and 95% CI) in CANTAB test outcomes are given for every 1-SD increase in CRAE, CRVE, or TI. Models were adjusted for age, sex, ethnicity, BMI, MAP, problem score, time at which the CANTAB test battery was initiated and season during which the follow-up visit took place. Complete data were available for 208 mother-child pairs. Abbreviations: BLC, Big/Little Circle; CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span; TI, Tortuosity index. *$p \leq 0.05$.

Supplemental Table S3. Sensitivity analysis additionally adjusting the multiple linear regression model between retinal vessel characteristics and outcomes of the four CANTAB tests, for MAP, season of follow-up, time of day at which the CANTAB test was performed, problem score, parental education level, smoking habits and alcohol use of the mother during pregnancy, and the exposure of the child to passive smoking of the parents

CANTAB test and outcome(s)	CRAE	CRVE	TI
MOT			
Mean latency	-0.29 (-3.74; 3.02)	2.16 (-1.18; 5.49)	-2.52 (-5.88; 0.84)
Mean accuracy	-0.14 (-0.58; 0.29)	-0.59 (-0.98; -0.18)*	0.24 (-0.24; 0.60)
BLC			
Mean latency	0.14 (-2.45; 2.88)	0.59 (-2.16; 3.14)	1.80 (-0.84; 4.44)
SSP			
Span length	-0.06 (-0.29; 0.13)	-0.14 (-0.39; 0.06)	-0.02 (-0.24; 0.12)
Mean latency of first response	3.31 (-2.88; 9.50)	3.14 (-2.94; 9.21)	3.12 (-3.00; 9.36)

Continued

Supplemental Table S3. Continued

CANTAB test and outcome(s)	CRAE	CRVE	TI
DMS			
Mean latency	0.72 (-4.46; 5.90)	0.59 (-4.51; 5.68)	6.48 (1.44; 11.52)*
Mean latency (trials with delays)	1.58 (-4.61; 7.92)	-1.37 (-7.45; 4.90)	8.04 (1.80; 14.16)*
Error given correct	1.44 (-1.58; 4.46)	2.35 (-0.59; 5.49)	-0.24 (-3.36; 2.76)
Error given error	1.73 (-1.15; 4.61)	1.96 (-0.98; 4.70)	-0.60 (-3.36; 2.28)
Percentage Correct	-1.44 (-3.60; 0.72)	-1.76 (-3.92; 0.39)	0.60 (-1.68; 2.76)
Percentage Correct (trials with delays)	-1.73 (-4.03; 0.58)	-1.57 (-3.92; 0.78)	-0.36 (-1.92; 2.64)

Estimated changes (% change and 95% CI) in CANTAB test outcomes are given for every 1-SD increase in CRAE, CRVE, or TI. Models were adjusted for age, sex, ethnicity, BMI, MAP, problem score, time at which the CANTAB test battery was initiated, season during which the follow-up visit took place, education level of both parents, maternal smoking and alcohol consumption during pregnancy and exposure of the child to passive smoking in its household environment. Education level was coded as low (no diploma or primary school), middle (high school diploma), or high (college or university degree). Maternal smoking status during pregnancy was defined as non-smokers, stopped smoking before pregnancy, and current smokers (smoked during pregnancy). Alcohol use of the mother during pregnancy was subdivided in mothers who did not consume alcohol during pregnancy, and mothers who consumed alcohol at least occasionally during gestation. Complete data were available for 188 participants. Abbreviations: BLC, Big/Little Circle; CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span; TI, Tortuosity index. *$p \leq 0.05$.

Supplemental Table S4. Sensitivity analysis excluding mothers who consumed alcohol during pregnancy (n = 50).

CANTAB test and outcome(s)	CRAE	CRVE	TI
MOT			
Mean latency	-1.01 (-4.18; 2.16)	4.12 (0.98; 7.25)*	0.24 (-3.12; 3.60)
Mean accuracy	-0.13 (-0.58; 0.29)	-0.39 (-0.78;-0.06)*	-0.08 (-0.48; 0.36)
BLC			
Mean latency	0.86 (-1.58; 3.31)	1.18 (-1.18; 3.53)	1.08 (-1.32; 3.60)
SSP			
Span length	0.04 (-0.12; 0.14)	-0.02 (-0.18; 0.14)	-0.01 (-0.12; 0.12)
Mean latency of first response	-0.10 (-5.47; 5.18)	1.18 (-4.12; 6.47)	2.40 (-3.00; 7.92)

Continued

Supplemental Table S4. Continued

CANTAB test and outcome(s)	CRAE	CRVE	TI
DMS			
Mean latency	-0.01 (-4.61; 4.61)	-0.39 (-4.90; 4.31)	3.24 (-1.44; 7.92)
Mean latency (trials with delays)	1.87 (-3.74; 7.34)	-0.98 (-6.47; 4.51)	5.04 (-0.60; 10.80)
Error given correct	1.01 (-1.73; 3.60)	2.35 (-0.18; 4.90)	1.08 (-1.56; 3.84)
Error given error	1.15 (-1.73; 3.89)	1.37 (-1.37; 4.12)	-0.48 (-3.36; 2.40)
Percentage Correct	-0.86 (-2.74; 1.15)	-1.76 (-3.53; 0.20)	-0.60 (-2.64; 1.32)
Percentage Correct (trials with delays)	-0.72 (-2.74; 1.15)	-1.18 (-3.14; 0.78)	-0.48 (-2.52; 1.56)

Estimated changes (% change and 95% CI) in CANTAB test outcomes are given for every 1-SD increase in CRAE, CRVE, or TI. Models were adjusted for age, sex, ethnicity, and BMI. Abbreviations: BLC, Big/Little Circle; CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span; TI, Tortuosity index. *p ≤ 0.05.

Supplemental Table S5. Sensitivity analysis excluding children with a problem score between 14 and 40 points (n = 33).

CANTAB test and outcome(s)	CRAE	CRVE	TI
MOT			
Mean latency	-0.58 (-3.60; 2.45)	2.55 (-0.59; 5.68)	-0.36 (-3.24; 2.52)
Mean accuracy	-0.07 (-0.43; 0.29)	-0.39 (-0.78; 0.02)	0.07 (-0.24; 0.48)
BLC			
Mean latency	1.15 (-1.01; 3.31)	1.57 (-0.59; 3.72)	1.32 (-0.72; 3.48)
SSP			
Span length	0.06 (-0.10; 0.14)	-0.04 (-0.20; 0.10)	-0.01 (-0.12; 0.12)
Mean latency of first response	-0.09 (-5.04; 4.90)	1.37 (-3.72; 6.27)	0.96 (-3.96; 5.76)

Continued

Supplemental Table S5. Continued

CANTAB test and outcome(s)	CRAE	CRVE	TI
DMS			
Mean latency	0.43 (-3.74; 4.61)	0.39 (-3.72; 4.70)	3.24 (-0.72; 7.32)
Mean latency (trials with delays)	1.01 (-4.03; 6.05)	-1.37 (-6.47; 3.72)	3.48 (-1.32; 8.40)
Error given correct	3.02 (0.43; 5.62)*	3.53 (0.98; 6.08)*	0.48 (-2.04; 3.00)
Error given error	1.44 (-1.01; 4.03)	1.57 (-1.18; 4.12)	-0.48 (-2.88; 2.04)
Percentage Correct	-1.87 (-3.74; 0.04)*	-2.16 (-4.12; -0.39)*	-0.05 (-1.92; 1.80)
Percentage Correct (trials with delays)	-1.58 (-3.60; 0.43)	-1.76 (-3.72; 0.12)	0.12 (-1.68; 2.04)

Estimated changes (% change and 95% CI) in CANTAB test outcomes are given for every 1-SD increase in CRAE, CRVE, or TI. Models were adjusted for age, sex, ethnicity, and BMI. Abbreviations: BLC, Big/Little Circle; CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span; TI, Tortuosity index. *$p \leq 0.05$.

Supplemental Table S6. Sensitivity analysis excluding children who were born preterm (gestational age < 38 weeks) (n = 11).

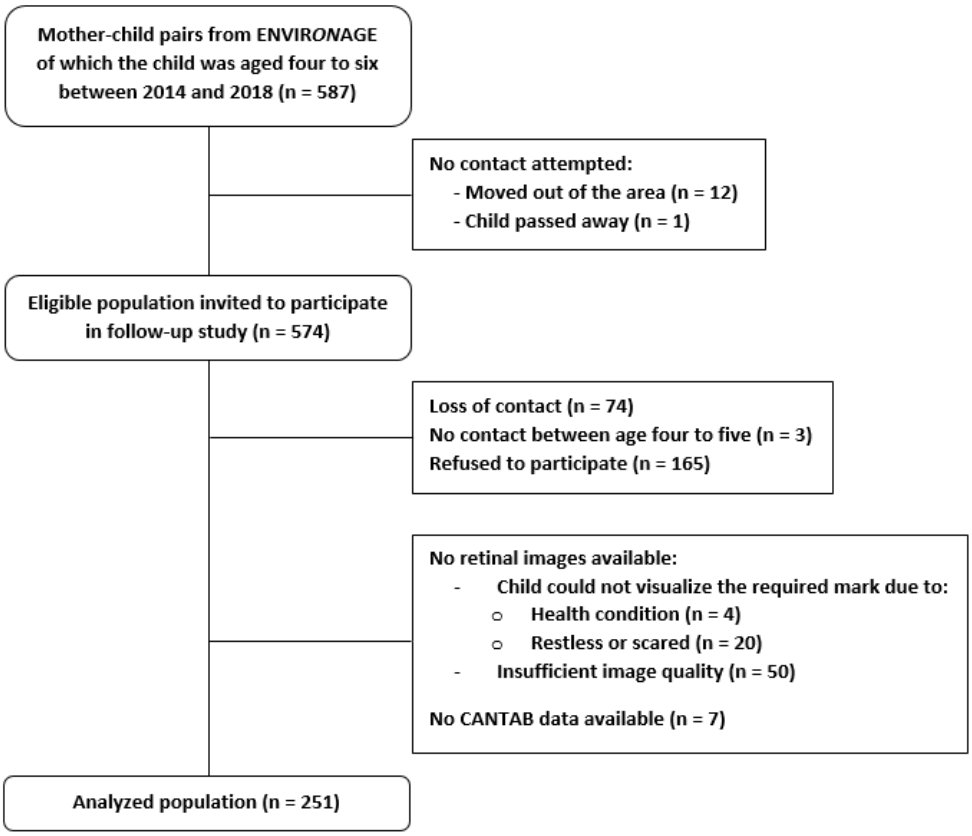
CANTAB test and outcome(s)	CRAE	CRVE	TI
MOT			
Mean latency	-1.30 (-4.18; 1.44)	3.14 (0.39; 6.08)*	-0.48 (-3.36; 2.28)
Mean accuracy	-0.09 (-0.43; 0.29)	-0.39 (-0.78; -0.08)*	-0.02 (-0.36; 0.36)
BLC			
Mean latency	0.43 (-1.73; 2.59)	1.18 (-0.98; 3.33)	1.08 (-1.08; 3.24)
SSP			
Span length	0.06 (-0.09; 0.14)	-0.04 (-0.18; 0.12)	-0.02 (-0.24; 0.12)
Mean latency of first response	0.13 (-4.61; 4.90)	1.18 (-3.72; 6.08)	0.48 (-4.08; 5.16)

Continued

Supplemental Table S6. Continued

CANTAB test and outcome(s)	CRAE	CRVE	TI
DMS			
Mean latency	-0.03 (-4.18; 4.03)	-0.06 (-4.31; 4.12)	3.12 (-0.96; 7.08)
Mean latency (trials with delays)	0.86 (-4.18; 6.05)	-1.76 (-6.86; 3.33)	4.32 (-0.60; 9.36)
Error given correct	2.02 (0.29; 4.46)	3.14 (0.59; 5.49)*	1.20 (-1.20; 3.48)
Error given error	1.30 (-1.15; 3.74)	1.18 (-1.37; 3.53)	0.24 (-2.16; 2.64)
Percentage Correct	-1.30 (-3.17; 0.43)	-1.76 (-3.53; 0.02)*	-0.72 (-2.52; 0.96)
Percentage Correct (trials with delays)	-1.44 (-3.17; 0.43)	-1.57 (-3.33; 0.39)	-0.36 (-2.28; 1.32)

Estimated changes (% change and 95% CI) in CANTAB test outcomes are given for every 1-SD increase in CRAE, CRVE, or TI. Models were adjusted for age, sex, ethnicity, and BMI. Abbreviations: BLC, Big/Little Circle; CRAE, Central retinal arteriolar equivalent; CRVE, Central retinal venular equivalent; DMS, Delayed matching to sample; MOT, Motor screening task; SSP, Spatial Span; TI, Tortuosity index. *$p \leq 0.05$.



<u>Supplemental Figure S1.</u> Flow chart summarizing the selection process resulting in the 251 participants of this study.

GENERAL DISCUSSION

The developmental origin of health and disease is a complex process, which we are only just beginning to unravel. Chronic diseases that arise in adulthood appear to originate early during fetal development, through an intricate interaction of a myriad of environmental exposures. Since the initial description of this hypothesis by Prof. Dr. David Barker, many studies have found molecular and clinical evidence describing the early development of diseases later in life, including investigations on the effects of ambient air pollution exposure during pregnancy. However, many pieces of the puzzle still need to be put in place, especially in terms of the integration of findings in different -omics areas and the clinical effects of prenatal air pollution exposure at the earliest stages of life.

In this doctoral dissertation, we investigated the effects of prenatal exposure to ambient air pollution and the effects on both the molecular level in placental tissue and on the clinical level, for the retinal microvasculature. We have established novel associations that aid in identifying the factors involved in disease development later in life, from the initial adverse exposure during gestation to the molecular placental biomarkers of this exposure and the associated clinical changes as markers for the effects on the microvascular system.

Novelties found in this dissertation include **(Figure 1)**:

- The establishment of an optimized protocol for the identification of the placental proteome
- The identification of differentially expressed placental proteins and associated enriched pathways for women exposed to high levels of black carbon during pregnancy.
- The identification of placental *TRPC6* as a novel marker of the effects of *in utero* ambient $PM_{2.5}$ exposure
- The description of the associations between $PM_{2.5}$ and NO_2 exposure and the retinal microvascular characteristics of 4-to-5-year-old children
- The establishment of the link between neurological developmental in children aged 4 to 5 years and their retinal microvascular traits.

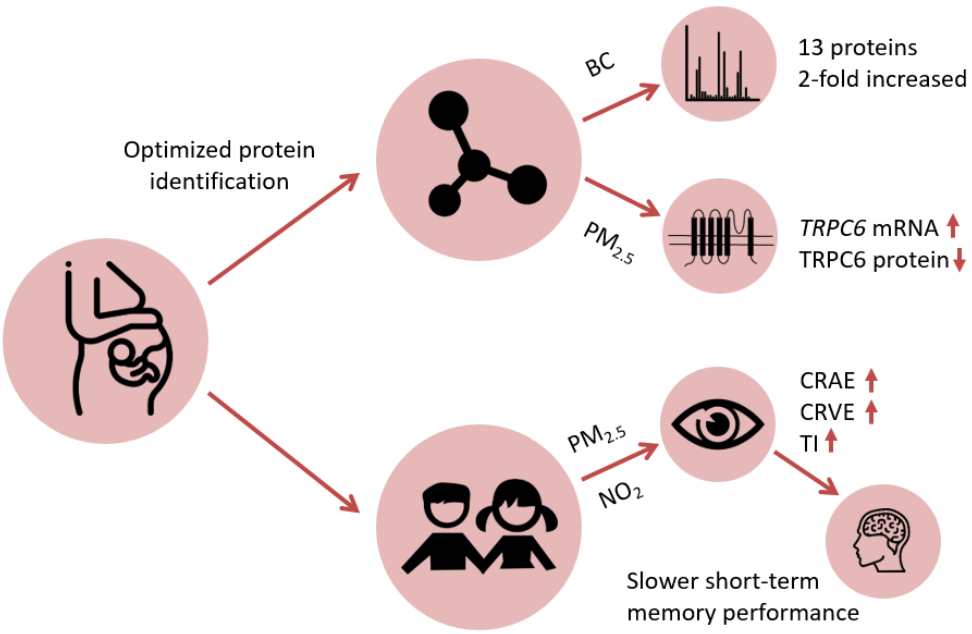


Figure 1. Summary of the findings described in this doctoral dissertation. Starting from exposure to ambient air pollution in the womb on the far left, we have studied 2 arms of effects: the upper arm represents the molecular effects in the placenta and the lower arm represents the clinical effects in children at the age of 4 to 5 years. The upper molecular findings arm shows the optimization of a protocol on the identification of placental proteins (**Chapter 1**), from which 2 other findings resulted: the identification of 13 proteins with a minimal twofold increased expression in the placentas of women exposed to high levels of black carbon air pollution during pregnancy (**Chapter 2**) and the identification of placental *TRPC6* mRNA and TRPC6 protein expression as potential markers of gestational $PM_{2.5}$ exposure (**Chapter 3**). The lower clinical findings arm follows 1 path with 2 subsequent connections. The first one describes the effects of both $PM_{2.5}$ and NO_2 air pollution exposure during pregnancy and the child's lifetime on an increment of retinal arteriolar and venular width (CRAE and CRVE, respectively) and of the retinal vessel tortuosity (TI) (**Chapter 4**). Finally, the second branch of the clinical findings arm described the effects of the retinal vessel characteristics on the neurocognitive development of the children in our ENVIR*ON*AGE cohort, more specifically a slower short-term memory performance in children with a higher microvascular width (**Chapter 5**).

This PhD project has several strengths and challenges. Apart from the summarized novelties, the greatest strength is that within the prospective ENVIR*ON*AGE birth cohort, currently counting over 2000 mother-child pairs, we could access a wealth of information on both mother and infant, from the moment of birth until the follow-up visit of the child. A difficulty in this dissertation, as for all epidemiological research, is taking into account confounding. For example, we took into account several potential confounding factors to unravel the influence of prenatal air pollution exposure on the microvasculature at age 4. A confounding variable is a factor that is associated with both the exposure and the outcome, without being an effect of the exposure itself.[249] For our statistical model, we selected several variables related to the child, such as age, sex, ethnicity, mean arterial blood pressure, BMI, the season in which the follow-up examination took place, birth weight, and the exposure of the child to passive smoking. Some factors related to the mother were taken into account as well, such as the age of the mother during pregnancy and her pre-pregnancy BMI, education level, alcohol use during pregnancy, and smoking habits before and during pregnancy.

With regard to the child, for example, age [250], sex [251] and ethnicity [252] are known to affect the development and characteristics of the (retinal) microvasculature. These factors are also known to influence how the effects of air pollution exposure manifest themselves in the human body. However, the exposure to air pollution itself does not have any effect on the sex, age, and ethnicity of a human being. Childhood BMI (and associated life style choices) can have an effect on the microvasculature and on the dispersion and extent of air pollution-related effects. However, a study conducted in Sweden concluded that prenatal exposure to air pollution did not affect the BMI of children at the age of 4.[253] The same reasoning can be used for active and passive smoking behaviour: cigarette smoke is known to have an effect on the development of the microvasculature, and is known to aggravate the effects of air pollution exposure, however air pollution levels will not determine the smoking behaviour of a mother during pregnancy, nor the exposure the child will have to passive smoking throughout life. Nevertheless, we take these factors into account based on *a priori* selection of both potential confounding factors and covariates.

1. The molecular burden of prenatal ambient air pollution exposure in the placenta: a multi-level effect

Over the past decades, evidence from both *in vivo* and *in vitro* studies has revealed that the effects of air pollution exposure during pregnancy manifest themselves on multiple levels. Starting in the lungs of the mother and progressing throughout the body and into the placenta, the direct and indirect effects of air pollution particles arise and initiate downstream effects in other tissues and organs. Recently, air pollution particles have been detected at the fetal side of the human placenta [34] which indicates that not just markers of indirect effects of air pollution exposure can be transported to or generated in fetal tissues, but also the particles themselves can translocate to these locations with potential detrimental effects for the fetus. Establishing molecular signatures of the (in)direct effects of air pollution exposure aids in tracing the effects of air pollution towards the fetus. Through the identification of molecular targets and pathways affected in tissues such as that of the placenta, we can put together how the fetus can be affected later in life through initial changes occurring before birth. Additionally, through the study of post-partum tissues such as the placenta, examining the effects of maternal exposures can occur in a non-invasive and safe way for the growing fetus, in contrast to the sampling of intra-uterine fluids, which always entails potential risks of pregnancy loss (although only to a very small extent) and stress for the mother.[254]

In the review paper established at the beginning of this doctoral project (**Appendix A**), it was summarized that placental molecular signatures of prenatal exposure to ambient air pollution have been identified on almost all -omics levels. However, there was a clear overrepresentation of markers identified on the level of the genome and the epigenome, while markers on the level of the transcriptome and proteome remain scarce. Another conclusion of this review was that all described markers of exposure were single targets or pathways, while no research in this regard had been performed on a top-down, whole -omic level.

Within this doctoral dissertation, we focused on placental molecular markers on two levels: mRNA expression and protein expression. By combining knowledge on multiple levels, we gave a more complete overview of the molecular effects of

$PM_{2.5}$ and black carbon air pollution exposure before birth. Moreover, besides studying separate markers of exposure on multiple levels, chosen via an *a priori* selection process based on pre-existing knowledge, we have described the effect of air pollution exposure during pregnancy on the entire placental proteome, providing the base for future directed downstream research projects.

1.1. Methodological considerations for placental proteomic research

Placental tissue is complex in its origin and composition, which translates to the complexity of analyzing its molecular structure and interactions. Techniques to identify placental characteristics on the mRNA level have been optimized over the past years, resulting in stable methods of RNA extraction, RNA purification and real-time PCR measurements, eventually translated in ready-to-use commercial kits. With screening methods for placental proteomics in mind, we found that there was no such thing as a standardized method for proteome analyses in placental tissue. Apart from the different amounts of tissue that were used for protein extraction in different research groups, we also noticed a discrepancy in extraction methods, digestions times, and several other factors that could influence the final number of protein identifications. Due to the complexity of placental tissue composition we believed that, especially for the sensitive analysis methods used in proteome research, a unified and standardized method of protein extraction and analysis would benefit the interpretability and comparability of placental proteome profiles, for studying both the placental tissue of healthy women and that of pathological pregnancies.

In **chapter 1,** we described an optimized method for the extraction of proteins from placental tissue, the subsequent digestion of the proteins into peptides and the final identification of the placental protein mixture. We put special focus on these areas since we believed that a standardized version of these steps was lacking and would be beneficial to unify results from different research groups in order to expand the knowledge on the placental proteome. Already very early in the process, we discovered that the removal of excess blood from the placental tissue biopsies before protein extraction proved to be very important for placental protein identification and therefore, we had also put an extra focus on this aspect.

From our results we could conclude that by washing placental samples thoroughly with a neutral substance such as PBS, we did not only reduce the number of blood-related protein identifications, such as albumine, but also increased the total number of identifications in our samples. Important to bear in mind is that the final number of proteins identified with nano LC-MS/MS is only a fraction of the total number of proteins actually present in a sample (**Figure 2**). When studying tissue-specific proteins, often low in both quantity and molecular weight, the reduction of blood-specific proteins can be of crucial importance, since a mass spectrometer can only spend a certain amount of time on peptide analysis and will therefore spend the most time on peptides that are highly abundant with a large molecular mass.[87] We are the first to prove that blood removal from placental tissue samples increases protein identification, and we recommend this step in all studies examining the placental proteome.

In terms of placental protein extraction, separation and digestion, we examined differences in the final number of identifications, in the percentage of non-specific cleavages and miscleavages, and in the percentages of proteins identified for different cellular locations. We finally recommended washing of the placental tissue samples in a neutral solution prior to protein extraction, followed by mechanical disruption of the tissue and FASP-based digestion into peptides, with a final analysis of the peptide mixture by nano LC-MS/MS. However, this would be the case for research goals in the same context as ours, namely obtaining an as high number of placental proteins as possible. For hypotheses on proteins related to the nucleus for example, we would advise 1D-gel separation of the protein extracts as well as a verification, and potentially a more specific optimization, of our technique for this particular purpose.

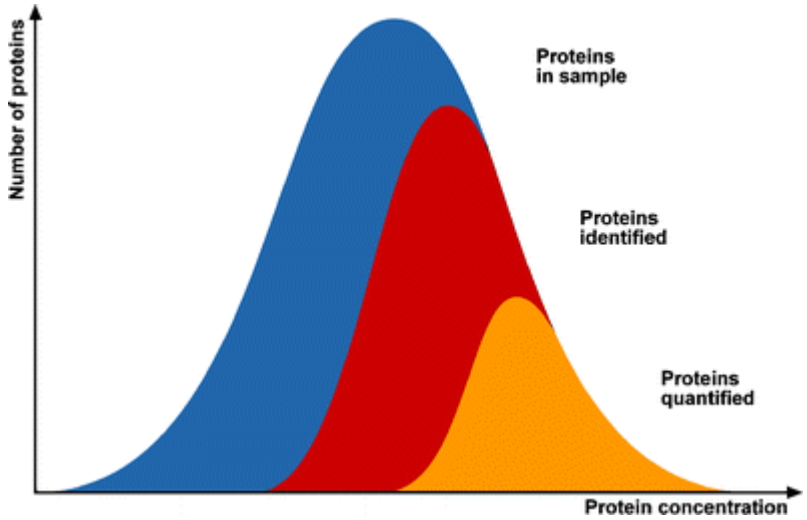


Figure 2. The final number of proteins identified and quantified with nano LC-MS/MS does not equal the total number of proteins present in a biological sample. A higher initial protein concentration results in a higher number of proteins identified and quantified, although the percentage of proteins quantified will always be only a fraction of the proteins identified and in turn, protein identification is never a complete representation of all proteins present in a cell or tissue. *Reprinted from Bantscheff; Analytical and Bioanalytical Chemistry.*[255]

Since it was recently described that the placenta consists of several sub-proteomes [85,256], depending on different locations in the entire organ, research groups observing these subclasses should verify if our optimized protocol is also the best fit to obtain high protein identification numbers for their research question. Additionally, we did not take into account the differences that could exist between the array of cell types found in the heterogeneous placenta. Varying in location and function, chorionic placental villi are composed largely of cytotrophoblasts and syncytiotrophoblasts, but also mesenchymal cells and mesenchymal-derived macrophages (also known as Hofbauer cells), fibroblasts, (vascular) smooth muscle cells, pericytes, and endothelial cells can be found in these villi.[257] The trophoblast sub-proteome has been studied in previous research [85,258], but we would advise this to be compared with the proteomes of the other key-player cell types of the placenta, and test whether our technique would also

be the advised methods to study these cellular sub-proteomes. For future placental proteomics research, we recommend that our method is revised whenever new, optimized techniques become available. However, we believe that removal of excess blood from placental tissue prior to protein extraction will remain of great importance to obtain a clear image of tissue-specific proteins. Furthermore, the final choice of method to digest the placental protein mixture into peptides will depend on the initial research hypothesis, and the choices that have to be made regarding costs and time. Especially when a choice needs to be made between a FASP-based or an in-gel digestion, these three points will prove essential for the final methodological choices.

1.2. Ambient black carbon air pollution exposure during pregnancy influences the placental proteome

Prenatal exposure to ambient air pollution can have a significant impact on the placenta, both on its morphological features and functioning, and on its molecular composition. Recent research by Bové and colleagues has shown that black carbon particles can be found at the fetal side of the placenta as early as 12 weeks of gestation.[34] This could be an indication that the BC particles themselves, apart from the indirect effectors caused by the presence of the particles, could have an effect on placental functioning, potentially affecting fetal development. The effects of BC exposure on the human placenta have not been studied extensively. Associations described to date include those on specific targets and pathways, such as DNA repair genes [99], placental 3-nitrotyrosine (3-NTp) as a marker of oxidative stress [26], and mitochondrial DNA methylation [98], but evidence on the relations between BC exposure and entire -omics fields is currently lacking.

In **chapter 2**, we have investigated the association between exposure to either low or high levels of ambient black carbon air pollution during pregnancy on the proteomic composition of the fetal side of the placenta. We found that, when a minimal 2-fold difference in expression was considered, 13 proteins were differentially expressed between the two BC exposure groups, all with higher levels in the placentas of the group of women exposed to high gestational black

carbon. Three protein-protein interactions were enriched within this list of 13 proteins, and functional pathway enrichment analysis put forward 5 involved pathways, namely the ECM-receptor interaction pathway, the regulation of sodium ion transport, intermediate filament organization, the formation of fibrin clots and hemostasis.

This is the first study on the effects of gestational BC exposure that identifies an array of different pathways affected in the placenta on the protein level. Some of the identified proteins and pathways, such as TIMP3 [120] and the ECM-receptor interaction and fibrin clot formation pathways [129,259], are also overexpressed in the placental tissue of mothers with preeclampsia, compared to their healthy counterparts. Since air pollution exposure during pregnancy has recently been identified as a potential risk factor for developing preeclampsia [121,122,260], this connection should be investigated in depth in future studies. Furthermore, upcoming research could focus on the importance of the protein-protein interactions specified within these pathways, to unravel the further consequences of an overexpression of these targets, both for the placenta and for the developing child in early life.

Pathway analysis showed that a wide variety of pathways are affected in the placenta in association with black carbon exposure during pregnancy. Systems related to (utero)placental vascular remodeling, such as the formation of fibrin clots and extracellular matrix receptor interaction, are upregulated, while proteins related to the mitochondrion are downregulated. This shows that the placenta is affected on various cellular levels in association with *in utero* air pollution exposure. Proper regulation of blood transport is pivotal for the development of the fetus, for the proper delivery of nutritional factors and hormones and the transport of waste substances to the mother. Although the formation of fibrin clots in the placenta increases towards the end of the third trimester, when the amount of clots accounts for over 5% of the placental weight this can become a pathological condition. When hemostatic malfunctioning causes the excessive formation and deposition of fibrin clots, this can lead to the occlusion of and infarcts in placental blood vessels, leading to placental vascular insufficiency.[261] Important to note here, is that none of the women participating in our study showed any complications related to pregnancy, nor was there any difference in

birth weight or birth length between the babies of the high versus the low BC exposure group. A good follow-up research hypothesis would be to investigate whether the placentas of women exposed to high levels of BC air pollution during pregnancy also have a higher percentage of fibrin clots and thus, if our findings on the proteome level would also reflect a clinical significance in this area.

Since ambient air pollution is more complex than just its black carbon fraction, we advise for future research on this topic that a selection in low and high prenatal exposure is also made for other air pollution fractions, such as $PM_{2.5}$, PM_{10} and ultrafine particles. By comparing, and potentially combining, the proteome (and other -omics) profiles from these exposures, a more general image of the effects of prenatal air pollution can be made, in turn guiding further research projects. Furthermore, we should look into the molecular and clinical associations of these findings, preferably already in children at an early stage in life. Therefore, it is again crucial that the children born from these pregnancies are followed in our cohort and others, to investigate the link between our identified placental proteome targets and the development of their health.

1.3. A placental (TRPC)6th sense? TRPC6 as a potential biomarker of $PM_{2.5}$ exposure before birth

Biomarkers of exposure, susceptibility and effect are important tools to describe the effects of environmental exposures on human health and on the development of chronic diseases later in life. In the placenta, several biomarkers associated with prenatal air pollution exposure have been identified in past research, such as specific miRNAs, mitochondrial DNA levels and markers of DNA damage.[97] However, since proper functioning of ion transporters, such as TRP channels, is crucial for the development of the placenta, and since earlier research in lung tissue has shown that TRP channels can be affected by exposure to air pollution [154], we wanted to investigate whether TRPC6, one of the most prominent TRP channels in the placenta, is also affected by prenatal air pollution exposure. We examined the association between gestational exposure to $PM_{2.5}$ ambient air pollution and the expression of the TRPC6 cation channel at the fetal side of the

placenta. We are the first to describe that *TRPC6* mRNA expression shows a 26.6% increment [95% CI: 12.1% to 43.0%; p = 0.0001] during gestational weeks 32 to 39 for every 5 µg/m³ increase in $PM_{2.5}$ exposure, as described in **chapter 3.** Moreover, we found a significantly decreased placental TRPC6 protein expression [-11.7%, 95% CI: -21.6% to -0.6%; p = 0.04] for the same increment in gestational $PM_{2.5}$ air pollution exposure, this time between weeks 3 - 15 and weeks 27 - 35 of pregnancy. Trimester-specific effects of environmental exposures on placental molecular composition have also been observed for other molecular targets. For example, a 15.9% decrease [95% CI): -28.7 to -3.2%; p = 0.02] in expression of brain-derived neurotrophic factor (*BDNF*) and a 24.3% decrease (95% CI: -42.8 to -5.8%; p = 0.011) in synapsin 1 (*SYN1*) expression is only associated with higher $PM_{2.5}$ exposure during the first trimester of pregnancy.[64] In relation to this, future research should pay more attention to the molecular pathways involved in specific actions within the different trimesters of pregnancy, and how these can be affected by environmental exposures such as ambient air pollution.

Since TRPC6 is a cation channel with a prominent role in the transport of Ca^{2+}, further investigations should be conducted on the effect of $PM_{2.5}$ on Ca^{2+} homeostatis in the placenta. Particularly during the third trimester of pregnancy, sufficient Ca^{2+} transport through the placenta is crucial for the formation of the fetal skeleton and proper fetal cellular functioning.[262] Another important finding of this work is that the expression of TRPC6 on the mRNA level was opposite to its expression on the protein level. Although both significant in association with third trimester $PM_{2.5}$ exposure, a positive relation was shown for *TRPC6* mRNA expression while a negative association was identified for TRPC6 protein expression. A discrepancy between both expression levels is not uncommon. Comparing RNA and protein levels between 23 human cell lines in the research of Gry and colleagues has revealed that a correlation between the two expression levels only existed for 1/3 of all screened targets.[140] Therefore, we would advise researchers studying associations between environmental stressors and molecular changes in the placenta to study these effects on different levels, since the conclusion from one level could potentially tell only half of the story, without integration on another molecular expression level of the same target. mRNA

expression and translation into proteins can be regulated on different intracellular levels. Firstly, RNA transcription can be prematurely ended by a specific structuring of the mRNA chain itself, also referred to as transcription attenuation. Secondly, the mRNA can be degraded by 5' exonucleases before it is translated into protein. The mRNA sequence can be protected from degradation by capping the 5'-ending with a methylated guanine nucleotide. This way, the sequence is marked for 5'-cap-dependent translation and can even be translated multiple times, depending on its stability. Similarly, the mRNA sequence can be protected from exonuclease degradation by polyadenylation, which adds a poly(A) tail to the 3'-end of the sequence. Thirdly, RNA splicing can also alter the translation of the mRNA sequence, leading to either the translation of alternative (forms of) functional proteins or to the loss of translation capability in case of the introduction of a stop codon into the sequence or loss of function of the protein itself by the spliced sequence. Post-transcriptional editing is another system that alters the translational meaning of an mRNA sequence. In humans, two types of mRNA editing have been recorded, namely the deamination of adenine to produce an inosine nucleotide and the deamination of cytosine to produce uracil.[263] Additionally, mRNA expression can be regulated by its interaction with specific micro RNA (miRNA) sequences. These short RNA sequences bind to their complementary mRNA strand, thereby marking the strand for translational silencing by immediate cleavage by or degradation in P-bodies.[264] Finally, regulation of RNA-binding proteins (RBPs), which are essential for correct translation of the mRNA sequence, can also be a level of control in the process from mRNA to protein. These RBPs are not only important to localize the RNA polymerase for transcription initiation, but also in the process of alternative splicing or transport of the mRNA sequence from the nucleus to the cytoplasm.[263]

Adding TRPC6 to the list of proteins identified to be differentially expressed in association with prenatal BC exposure in the STRING protein-protein interaction and pathway analyses, did not show a significant interaction with any of the proteins significantly over- or under expressed in association with prenatal BC exposure. However, several common cellular processes are shared between TRPC6 and other identified targets, such as platelet activation and coagulation, response to both wounding and cellular stress, regulation of (metal) ion transport

and regulation of body fluid levels. Therefore, although TRPC6 does not have a direct interaction with any of the identified targets, a functional connection in the setting of prenatal air pollution exposure could be present. However, the function and relations of TRPC6 in this context should be further investigated.

2. The eye as a window to the brain: air pollution exposure and the retinal microvasculature

The effects of ambient air pollution exposure on the cardiovascular system have been described extensively over the past years, with the largest evidence found on blood pressure [77], the development of cardiovascular disease [265], and the chances of having a stroke.[266] With our research, discussed in **chapter 4,** we have not only shown that air pollution exposure has a distinct effect on the microvasculature, but also that this already manifests itself at a very young age. Moreover, we have found that both $PM_{2.5}$ and NO_2 exposure via the mother during pregnancy, and direct short-term and long-term exposure to $PM_{2.5}$ and NO_2 of the children during their lifetime can have an effect on the microvasculature of the retina.

In our study, for every IQR increase in $PM_{2.5}$ or NO_2 exposure during the whole pregnancy, the changes in retinal vessel diameter ranged from a 2.87 µm widening of the arteriolar diameter for prenatal $PM_{2.5}$ exposure to 4.03 µm widening of the retinal venules for gestational NO_2 exposure. The clinical effects of retinal vascular widening have not yet been studied in children, and only in very few studies on adults. Within our ENVIR*ON*AGE cohort, we found that a decrease in arteriolar diameter was correlated with higher systolic blood pressure ($r = -0.12$; $p = 0.07$) and diastolic blood pressure ($r = -0.19$; $p = 0.0036$), although no significant correlations were described between the venular diameter and any of the macrovascular parameters.[267] A study in 10,911 adults has found that widening of the retinal venules increased the chances of suffering an abdominal aortic aneurysm (hazard ratio 1.61), between the patients with the lowest (127–182 µm) and the highest (204–276 µm) quartile of retinal venules.[268]

Air pollution is a complex mixture of substances, both solid and gaseous in nature. The complexity and heterogeneity of air pollution makes it challenging to differentiate between the effects of the separate components on the development of molecular and clinical characteristics. Moreover, since ambient conditions always imply that an organism is exposed to the air pollution mixture, rather than a single component, differentiating between the effects of single components not always tells the entire biological story. Since NO_2 is known to have a distinctive effect on the cardiovascular system, we wanted to verify whether this separate component of air pollution in itself could be associated with retinal microvascular outcomes at age 4. In our cohort, $PM_{2.5}$ and NO_2 were strongly and positively correlated for the whole pregnancy (Pearson $r = 0.72$, $p < 0.0001$) and for all 3 trimesters of pregnancy ($r = 0.71$ for trimester 1, $r = 0.78$ for trimester 2 and $r = 0.78$, for all $p < 0.00001$). A similar magnitude for the correlation between $PM_{2.5}$ and NO_2 exposure levels were found for other studies and cohorts as well.[269–271] Since there is such a strong linear relationship between these 2 exposures, collinearity is very likely occur by adding them into the same statistical equation, affecting the size of the other regression estimates, and making the distinction of separate air pollution subset effects statistically challenging.[272] Recently, one of the first attempts to study the fractionated effects of air pollution was performed by Nováková and colleagues.[273] However, even in these controlled laboratory conditions, it is very difficult to study the effect of each of the separate components individually.

The greatest focus of future research on this topic should be twofold. Firstly, it should be verified that the observed changes in vessel width and tortuosity persist over time. Although we have taken into account exposure over a large period of time (i.e. 9 months of pregnancy and a life span from birth until 4 to 5 years of age), the cardiovascular system is an ever changing and adapting system, and can be influenced by a number of factors apart from air pollution exposure such as physical activity, and eating pattern. A study on a population of healthy volunteers between 20 and 69 years described an age-dependent loss of myogenic response in retinal arterioles.[274] However, more research on retinal vascular ageing, and the consequences of air pollution thereof, is certainly needed.[275] Secondly, the relation between changes on the microvascular level and the

potential consequences on the macrovascular level should be further examined. Since there is still an apparent lack of research on the macrovascular effects of microvascular (retinal) alterations, especially in children, upcoming studies should verify whether the changes in vessel width and tortuosity in children could be a predicting factor for their cardiovascular and general health development later in life.

2.1. The role of retinal vasculature and prenatal air pollution exposure in neurocognitive development during childhood: a complex story

Recently, Wei and colleagues have shown that in a population of 11-year-old children, cognition was associated with the microvasculature characteristics of the retina.[199] In our research, discussed in **chapter 5**, we have shown that this relation can be determined a lot earlier in life, already at an age between 4 and 5 years. More specifically, we have found that at this age, short-term visual recognition memory performance and not attention, psychomotor speed or visuospatial working memory capacity is associated with a child's retinal arteriolar and venular width and vessel tortuosity. This was represented by 1.76% (95% CI: −3.53%to −0.04%; p = 0.04) fewer correct DMS assessments, and 2.94% (95% CI: 0.39 to 5.29; p = 0.02) more errors given a previous correct answer in the DMS task, for every 1-SD widening in CRVE. A 1-SD widening in CRAE increased the total percentage of errors and errors given previous correct answers in the DMS task with 1.44% (95% CI, −3.25% to 0.29%; p = 0.09) and 2.30% (95% CI, −0.14% to 4.61%; p = 0.07), respectively. A 1-SD higher vessel tortuosity was associated with a 4.32% relative increase in latency in DMS task performance (95% CI, −0.48% to 9.12%; p = 0.07).

Since only very few research on this topic has been conducted, it is difficult to estimate whether the effects we have assessed in association with a higher retinal vessel width will persist in time and if these changes on such a young age will cause a significant outcome for the overall neurocognitive development of the child. Only 2 other studies on the use of the CANTAB test battery have been published, confirming both the efficacy and the difficulty of performing these tasks in children within this age group.[230,248] Moreover, the consistency of the tests in

our CANTAB test battery at such a young age needs to be verified. In a Finish group of elementary school children with an average age of 12.2 years, the 1-year consistency of for example the SSP test was found to be moderate (r = 0.37, p = 0.001) [276], which certainly has to be taken into account for further testing in our prospective ENVIR*ON*AGE cohort. The IQ score of children is known to fluctuate over time, and only becomes more stable at a later age. Therefore, as is the case for the microvascular changes in the retina, it is important that this test battery is repeated within the children of the ENVIR*ON*AGE follow-up cohort, to evaluate whether these changes persist over time.

In contrast to what we expected, we did not find an association between gestational exposure to $PM_{2.5}$ and neurocognitive development within the ENVIR*ON*AGE follow-up cohort (results not discussed within this doctoral dissertation), although we found an association between prenatal $PM_{2.5}$ exposure and the retinal microvasculature, and an association between the retinal vessel width and short-term memory functioning. Recent research by Chen and colleagues describes that prenatal $PM_{2.5}$ exposure already affects the neurological development of neonates, namely via a negative relation with both behavior and activetone score, assessed 48–72 h postpartum with the neonatal behavioral neurological assessment (NBNA).[270] In our cohort, an analysis on the mediatory effect of $PM_{2.5}$ in the relation between the retinal blood vessels and the results of the CANTAB test battery, did not show a mediatory effect of whole pregnancy $PM_{2.5}$ exposure on the association between a widening of the retinal venules and the results of the DMS task. Since we have only started to unravel this intricate relationship between the retinal microvasculature, air pollution exposure, and the effects on neurological development during childhood, taking into account a potential mediatiory effect, this should be further explored in depth. For example, it can be explored whether other fractions of air pollution, such as BC, NO_2 or UFPs act as a mediator between retinal vessel characteristics and neurological test outcomes at the age of 4 to 5 years and later in life.

3. From maternal exposure to child development: how the placenta can become a mediator for health effects later in life

The methods through which molecular changes in placental tissue translate into clinical effects in the neonate are complex and multifold. Over the nine-month period of gestation, a myriad of signals is sent from the mother to the developing fetus, although the exact combination and sequence of these signals are currently unclear. Especially in the beginning of pregnancy, the most important communication originates from the ovaries and uterus, through for example hormones such as estradiol and progesterone. Later on, factors originating from the maternal blood circulation, such as interleukins, prolactin and leptin, get a larger role in maternal-fetal signaling.[277] The placenta itself is also capable of producing leptin, and recent research has shown that factors such as prenatal air pollution can alter the placental methylation pattern of the leptin gene sequence.[62] Research on pathological conditions such as preeclampsia reveals that the process translating maternal conditions and exposures to effects in the fetus is more intricate than a single system malfunction.[278] Apart from a role for genetic predisposition and comorbidities such diabetes and cardiovascular disease, unsuccessful placentation and subsequent oxygen deficiency and oxidative stress can also influence the development of preeclampsia. In this regard, factors originating from the placental tissue itself, such as placental growth factor (PlGF), have been identified as biomarkers for the diagnosis and prediction of this adverse health condition.[279] The production of PlGF is crucial for proper angiogenesis, and hence proper development, of the placenta throughout pregnancy. In a transgenic mouse model, PlGF overexpression often resulted in loss of the pups, and viable pups had severely disrupted development of their cardiovascular system and were restricted in growth.[280] Another example is SERPINE1, in our research found to be overexpressed in the placenta in association with maternal exposure to ambient black carbon during pregnancy. In mothers with various adverse pregnancy conditions such as preeclampsia, this protein is overexpressed in the maternal blood stream, as well as in placental tissue.[281,282]

An interesting example of maternal-fetal communication and the role of the placenta therein, is the identification of the brain-placental axis. This communication goes in two directions.[283] Firstly, hormones produced by the

placenta, such as placental serotonin, melatonin, and oxytocin, can influence the maternal physiology, including that of the brain, with the purpose to sustain the pregnancy and prepare the mother for lactation and protection when the child is born.[284] Secondly, the placenta plays a role in the development of the fetal brain. Again, this can be regulated by placental hormone production, but also by changes in the placental expression of molecular pathways involved in angiogenesis and growth. For example, silencing of insulin-like growth factor-2 (IGF-2) in placental tissue of mice results in a greater sense of anxiety in the offspring, potentially by inducing intrauterine growth restriction and reducing the total amount of nutrients delivered from mother to fetus.[285]

Within this project, we established the link between (prenatal) air pollution exposure and microvascular outcomes at the age of 4. One of the focusses of future research should be put on the placental biomarkers that could be mediators of this exposure in establishing a clinical outcome early in life. In this context, PlGF, equal in function to the more widely expressed vascular endothelial growth factor (VEGF), can play an important role. In a cohort of 3505 children, lower levels of PlGF in the second trimester of pregnancy were associated with a narrower retinal arteriolar diameter at the age of 6.[286] Furthermore, decreased PlGF levels, as well as lower pregnancy-associated plasma protein-A (PAPP-A) levels, in maternal serum samples taken at the end of the first trimester were associated with a higher incidence of adverse cardiovascular conditions in the child at birth.[287] The receptor of PlGF, soluble fms-like tyrosine kinase-1 (sFlt-1), has an important role in the vascularization process as well. Apart from a broadly described role in preeclampsia, with the sFlt-1/PlGF ratio already used as a marked to establish preeclampsia in an early stage of the condition, increased expression of this receptor has been associated with a coronary heart disease and heart failure.[288,289]

Placental hypoxia is also known to affect cardiovascular development later in life. In a mouse model, the placental expression of 4 genes known for their role in hypoxia, Dio3, Hif3α, Pck1 and Hsd11b2, was increased in mice with late-gestation hypoxia and their offspring showed a significant increase in their blood pressure and heart rate.[290] The latter of these 4 placental markers, 11β-HSD2, is in humans also a well-established preventer of fetal overexposure to glucocorticoids of

maternal origin, and its expression was negatively associated with systolic blood pressure in the children at the age of 1.[291] Furthermore, measuring the expression and circulating levels of molecules associated with the immune and cellular stress defence system would be an asset in the study of air pollution effects on (micro)vascular development. For example, interleukin 33 (IL-33) has been associated with cardiovascular disease incidence and in placental tissue, macrophage-derived IL-33 was shown to be important for triggering trophoblast proliferation and was activated by cigarette smoke exposure.[292,293]

Finally, within this project, we identified several proteins in the placenta affected by prenatal black carbon exposure, which could be linked to the development or functioning of vascular pathways. Investigating these targets, for example ATP2B4, GP1BB, SERPINE2, and TIMP3, in the relationship between air pollution exposure during pregnancy and microvascular development in the children of our cohort would verify whether these complete the story from prenatal exposure, to placental mediator, and final clinical microvascular effect. Taken together, measuring these placenta-derived hormones, immunoregulatory molecules and tissue biomakers in our cohort and investigating the potential mediatory link of these targets between *in utero* air pollution exposure and the retinal vasculature, would be a great asset in further elucidating this relationship.

3.1. Placental molecules affected by air pollution exposure and their link with cognition

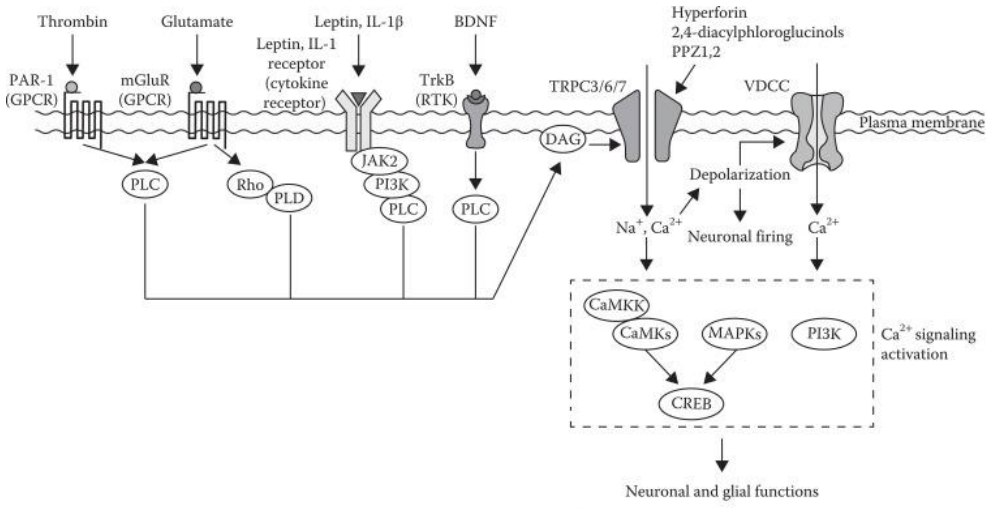
Several of the placental proteins identified to be differentially expressed in association with gestational BC exposure in our work, can be linked to neurocognitive functioning and development. Cognitive dysfunction in disease settings, such as Parkinson's disease, clinical depression and schizophrenia, has been associated with the overexpression of STAT1, one of the proteins with significantly increased expression in placental tissue of the high prenatal BC exposure group.[294–296] The function of this protein has also been described in normal neurogenesis and neurological functioning. Increased binding affinity of STAT1, induced by IFN-γ, is needed for proliferation and differentiation of neuronal stem cells.[297] The inhibition of STAT1 functioning in the JAK2/STAT1

pathway on the other hand has been shown to reduce cognitive decline in a senescence-accelerated mouse model.[298] Another important regulator of neurocognitive functioning is tissue inhibitor of matrix metalloproteinase-3 (TIMP3). A knockout mouse model for this protein shows an apparent decrease in cognitive abilities assessed via the water maze test, and both *in vivo* and *in vitro* experiments have pointed out that TIMP3 has a neuroprotective effect, especially in the hippocampus, by activating the Akt-mTORC1 pathway and with this action promoting neurite outgrowth.[299,300] Collagen type 4 alpha-2 chain (COL4A2) expression changes have mostly been associated with cognitive dysfunctions related to vascular abnormalities in the brain. In patients with cerebral small vessel disease, and related vascular cognitive impairment, both studies using whole genome and whole exome sequencing techniques have identified COL4A2 as a biomarker for this condition.[301,302] In a study on patients with COL4A1/COL4A2 mutations, several severe neurological disorders were identified, such as focal seizures and epilepsy, in 46.4% of the cases associated with the occurrence of porencephalic cysts.[303]

Several of the differentially expressed placental proteins in relation to gestational black carbon exposure, have been linked to the pathological profile of Alzheimer's disease in the context of cognitive functioning and decline later in life. Air pollution exposure is known to trigger inflammation and Alzheimer-like pathological signatures in the brain, and even during early childhood these molecular signals begin to manifest themselves.[304,305] Although mostly known as marker of kidney function, cystatin C (CYTC) seems to play a role especially in the neurovascular aspect of neurological conditions such as (vascular) dementia and Alzheimer's disease. Even mild cognitive development has been associated with a higher levels of cystatin C in serum samples.[306] Increased levels of CYTC in rats suffering from cerebral microbleeds had a negative effect on cognitive function, potentially by inhibiting the ERK/synapsin pathway.[307] In contrast, decreased serum levels of CYTC were found to be associated with a higher chance of developing Alzheimer's disease.[308] In placental tissue, cystatin C levels were increased in pathological conditions such as preeclampsia, and thus far, no studies have examined the impact of decreased CYTC expression in the placenta.[309] Another marker of Alzheimer's disease which showed an increased placental expression with high

prenatal BC exposure is serum amyloid P-component (APCS). This protein was already identified as a marker for Alzheimer's in the early 90's and is a major component of amyloid plaques, although serum levels of this protein are significantly decreased in people suffering from mild cognitive impairment.[310,311] Increased expression of ATP synthase subunit a (ATP6) has been associated with the early onset of Alzheimer's disease.[312] It is suspected that, through a disruption of mitochondrial oxidative phosphorylation components, such as ATP6, the oxidative balance is tilted towards ROS production and oxidative stress, eventually resulting in neurological damage and dysfunction. Furthermore, mutations leading to dysfunctional ATC6 in mitochondria have been associated with several serious pathological conditions, mostly of neurological nature, such as cognitive dysfunction, neuropathy, ataxia, seizures, and retinopathy.[313] Solute carrier family 2 facilitated glucose transporter member 3 (SLC2A3), encoding glucose transporter-3 (GLUT3), has a decreased expression in people with both apparent and asymptomatic Alzheimer's disease, and a decrease in GLUT3 was also associated with increased severity of amyloid plaque deposition.[314] Some copy number variants of this gene were also found to be linked to attention-deficit/hyperactivity disorder ADHD. A meta-analysis conducted by Merker and colleagues showed that the Rs12842 single nucleotide polymorphism (SNP) in the studied population of ADHD patients was associated with increased *SLC2A3* expression in peripheral blood cells and with deficits in working memory and cognitive response control.[315] Furthermore, when mouse hippocampal cells were exposed to hyperglycemic conditions, increased GLUT3 expression resulted in these cells, as well as increased apoptosis biomarkers and decreased levels of brain derived neurotrophic factor (BDNF) and synaptophysin (SYN).[316] Another *in vitro* setting has shown that when GLUT3 expression is blocked, the impaired glucose uptake prevents the normal outgrowth of neurite connections.[317] BDNF regulation in Alzheimer's disease is also regulated by another target identified in this project, SERPINE1. In the Tg2576 mouse model of Alzheimer's disease, increasing SERPINE1 levels resulted in more efficient BDNF maturation, avoiding apoptosis in neurons by high BDNF precursor levels, and better neurological functioning.[318]

TRPC6 is not only expressed in the placenta: this TRP channel also exerts an important function in neurological tissue. In rats, TRPC6 activation has been found to protect the brain against ischemic damage.[319] Granule cell neurons have been found to degenerate following TRPC6 knockdown, and TRPC6 activation results in increased Lon protease-1 (LONP1) expression and extracellular-signal-regulated kinase 1/2 (ERK1/2) phosphorylation, leading to a neuroprotective effect by triggering the destruction of dysfunctional mitochondria, thereby protecting the redox balance in the cell.[320] The role between TRPC6-triggered ERK1/2 activation has been established in neuronal tissue, but this link has not yet been studied in the placenta. Since placental tissue is able to produce leptin, and since leptin receptor activation can lead to an increase in PLC and DAG, this could lead to the activation of TRPC6 and hence an increased influx of Ca^{2+} into the cell, since this complex sequence of relations has already been established in the brain (**Figure 3**).(175)



<u>Figure 3.</u> Neuronal activation of TRPC6 channels and the influence of neighbouring transmembrane systems. Activation of cytokine receptors by factors such as leptin and BDNF can trigger the activation of the PLC pathway, the subsequent production of DAG and the activation of TRPC6 and other TRPC channels. Increased Ca^{2+} influx resulting from this action can cause neuronal depolarization and firing. *Reprinted and adapted from Sawamura et al; Neurobiology of TRP Channels.*[175]

4. Future perspectives: advice for follow-up research and legislations

This doctoral dissertation focused on two important aspects of air pollution exposure during pregnancy, namely the early molecular effects in the placenta, and the clinical effects early in life. We found that prenatal exposure to air pollution can affect both the protein composition of the placenta and the microvascular traits of children aged of 4 to 5 years. Furthermore, we described that an optimized and standardized method can increase protein identification in placental proteome analysis. Based on the findings of this work, we have several directions and recommendations for future work in this research area.

4.1. Let's unify and amplify: the need for integration of -omics level findings and bottom-up and top-down research

From our research, we can conclude that for a single target there can be a substantial difference between expression on the mRNA and protein level. If we would have only studied the expression of TRPC6 on either the mRNA or the protein level, our conclusion would have been incomplete and would have given a distorted view on the true effects of gestational $PM_{2.5}$ exposure on TRPC6 expression at the fetal side of the placenta. Therefore, we believe that future research should focus on the integration of the results found on different –omics levels, for thorough and structured conclusions to be made on the effects of gestational air pollution exposure on an organ such as the placenta.

Secondly, we have described the effects of air pollution exposure during gestation both on a single *a priori* determined target, TRPC6, and on the level of the placental proteome, identifying several important pathways associated with gestational BC exposure. Since we were the first to identify these relations in both cases, we advise that future research should further examine these alterations in depth, both bottom-up for the interaction partners of TRPC6 and top-down for the placental pathways found to be affected by BC exposure during pregnancy. TRPC6 is an important cation channel with a great affinity for the transport of Ca^{2+}. Measuring Ca^{2+} levels in term samples of the fetal side of the placenta and investigating the association with both TRPC6 expression and $PM_{2.5}$ exposure

during the third trimester of gestation would provide a more complete image on the effects of this environmental exposure during pregnancy.

Thirdly, we would recommend that our findings on the effects of $PM_{2.5}$ and NO_2 air pollution on the microvasculature in 4-to-5-year-old children are further tracked and analysed at later stages in life. By measuring retinal microvascular parameters in the same population during multiple follow-up visits at a later age, we could determine whether the changes seen at such a young age persist throughout life. Eventually, the development of cardiovascular conditions in adulthood can be monitored and studied in association with the microvascular changes at early childhood. In this regard, the levels of air pollution throughout life should be continually monitored, but the evolution of general health, physical activity and nutritional patterns should also be mapped and followed as potential effectors of micro- and macrovascular health.

4.2. European and worldwide legislation on air pollution: conclusions from evidence and paths to follow

In **Chapters 2, 3 and 4**, we have discussed the effects of exposure to ambient air pollution on both molecular and clinical outcomes at a very early stage in life. The first component of air pollution we discussed in **Chapter 2** is black carbon. We found an association between high BC exposure during pregnancy and 59 1.5-fold differentially expressed proteins in placental tissue, summarizing several affected pathways. With regard to the Belgian, European and WHO limitations and legislation on black carbon pollution, we can simply say that on all three levels, there is none. The WHO provides a report with the results of a systematic review on the effects of BC exposure on human health. The authors conclude that short-term BC exposure is a better indicator of adverse (traffic combustion-related) health effects than PM exposure in general, and that a reduction in the levels of $PM_{2.5}$, containing BC, would reduce the effects associated with PM exposure. In addition, they summarize that BC itself would not be the major toxicant, but it would provide a carrier surface for particles with a greater effect on human health.[321] However, since we found in **Chapter 2** that with a difference in average

exposure of 1.12 µg/m³ between the high and low BC exposure group, there was already a very significant effect on placental protein expression, and since other evidence from literature indicates that black carbon exposure can have a great influence on human health, we would advise that limitation guidelines would be put in place and discussed on both a national and international level.

The second and third component of ambient air pollution we focussed on in this dissertation were $PM_{2.5}$ and NO_2. In association with increased exposure levels during pregnancy, we have described both an increment in *TRPC6* mRNA expression in placental tissue for $PM_{2.5}$ exposure, and an increment of the retinal arteriolar and venular diameter with an increment in both $PM_{2.5}$ and NO_2 exposure. As described in the introduction of this dissertation, emission limits and legislations have been put in place on all regulatory levels. For $PM_{2.5}$, the average annual exposure level for the EU was set at 25 µg/m³, and at 10 µg/m³ for the WHO. The European limit for NO_2 exposure is equal to that of the WHO, namely an average annual exposure of 40 µg/m³. Since the molecular and clinical effects for our studies can already be perceived for much lower levels than the limits described by the EU, more specifically a maximum average weekly exposure of 16.6 µg/m³ $PM_{2.5}$ for our study on *TRPC6* expression and a mean exposure of 14.3 µg/m³ $PM_{2.5}$ and 19.7 µg/m³ NO_2 over the entire pregnancy for our study on the retinal microvasculature, we would strongly recommend both instances to revise their legislation based on the scientific evidence known today. The clinical and molecular effects described in this project point out that already at a very young age, and even before life outside the womb is initiated, the detrimental effects of air pollution can manifest themselves and that hence, better sooner than later, legislatory instances should focus on the most vulnerable parts of society when making decisions for our environment of the future.

APPENDIX A

APPENDIX A

AIR POLLUTION AND THE FETAL ORIGIN OF DISEASE: A SYSTEMATIC REVIEW OF THE MOLECULAR SIGNATURES OF AIR POLLUTION EXPOSURE IN HUMAN PLACENTA

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APPENDIX A

ABSTRACT

Background: Fetal development is a crucial window of susceptibility in which exposure-related alterations can be induced on the molecular level, leading to potential changes in metabolism and development. The placenta serves as a gatekeeper between mother and fetus, and is in contact with environmental stressors throughout pregnancy. This makes the placenta as a temporary organ an informative non-invasive matrix suitable to investigate omics-related aberrations in association with *in utero* exposures such as ambient air pollution.

Objectives: To summarize and discuss the current evidence and define the gaps of knowledge concerning human placental -omics markers in association with prenatal exposure to ambient air pollution.

Methods: Two investigators independently searched the PubMed, ScienceDirect, and Scopus databases to identify all studies published until January 2017 with an emphasis on epidemiological research on prenatal exposure to ambient air pollution and the effect on placental -omics signatures.

Results: From the initial 386 articles, 25 were retained following an *a priori* set inclusion and exclusion criteria. We identified eleven studies on the genome, two on the transcriptome, five on the epigenome, five on the proteome category, one study with both genomic and proteomic topics, and one study with both genomic and transcriptomic topics. Six studies discussed the triple relationship between exposure to air pollution during pregnancy, the associated placental -omics marker(s), and the potential effect on disease development later in life. So far, no metabolomic or exposomic data discussing associations between the placenta and prenatal exposure to air pollution have been published.

Conclusions: Integration of placental biomarkers in an environmental epidemiological context enables researchers to address fundamental questions essential in unraveling the fetal origin of disease and helps to better define the pregnancy exposome of air pollution.

INTRODUCTION

Both genetic and environmental factors contribute to a multitude of complex diseases, while the precise environmental causes and early pathophysiological mechanisms of these diseases remain poorly understood.[322] The development of diseases can find its origin in every stage of human life. However, the distinct time windows, i.e. pregnancy, infancy, adolescence, adulthood, and old age are characterized by differences in age-specific susceptibilities.[323] During the last decade, a major public health concern has focused on the pregnancy period during which the exposure to harmful substances should be avoided to give the newborn the chance to start life as healthy as possible.[324]

Over the entire intrauterine period, the placenta plays a crucial role for growth, development, and survival of the fetus.[38] After the syncytiotrophoblast cells of the blastocyst have invaded the uterine wall, the placenta starts to grow with the formation of chorionic villi, which constitute the fetal side of this temporary organ (**Figure 1**). One of the first functions of placental cells is to suppress the maternal immune system in such a way that the developing embryo is not rejected.[39] In later stages of pregnancy, the placenta develops a wide spectrum of functions to ensure proper fetal growth. It is endowed with an important transport function mediating the transfer of oxygen, nutritional components, growth factors, and hormones from mother to child, while carbon dioxide and other waste substances are transferred in the opposite direction.[40] This may occur by means of simple diffusion, (energy driven) transporter proteins, and endo- or exocytosis within complex matrices of different cell types, such as trophoblasts, amniotic cells, endothelium lining of the placental blood vessels, decidual cells, Hofbauer cells, and mesenchymal cells.[38]

APPENDIX A

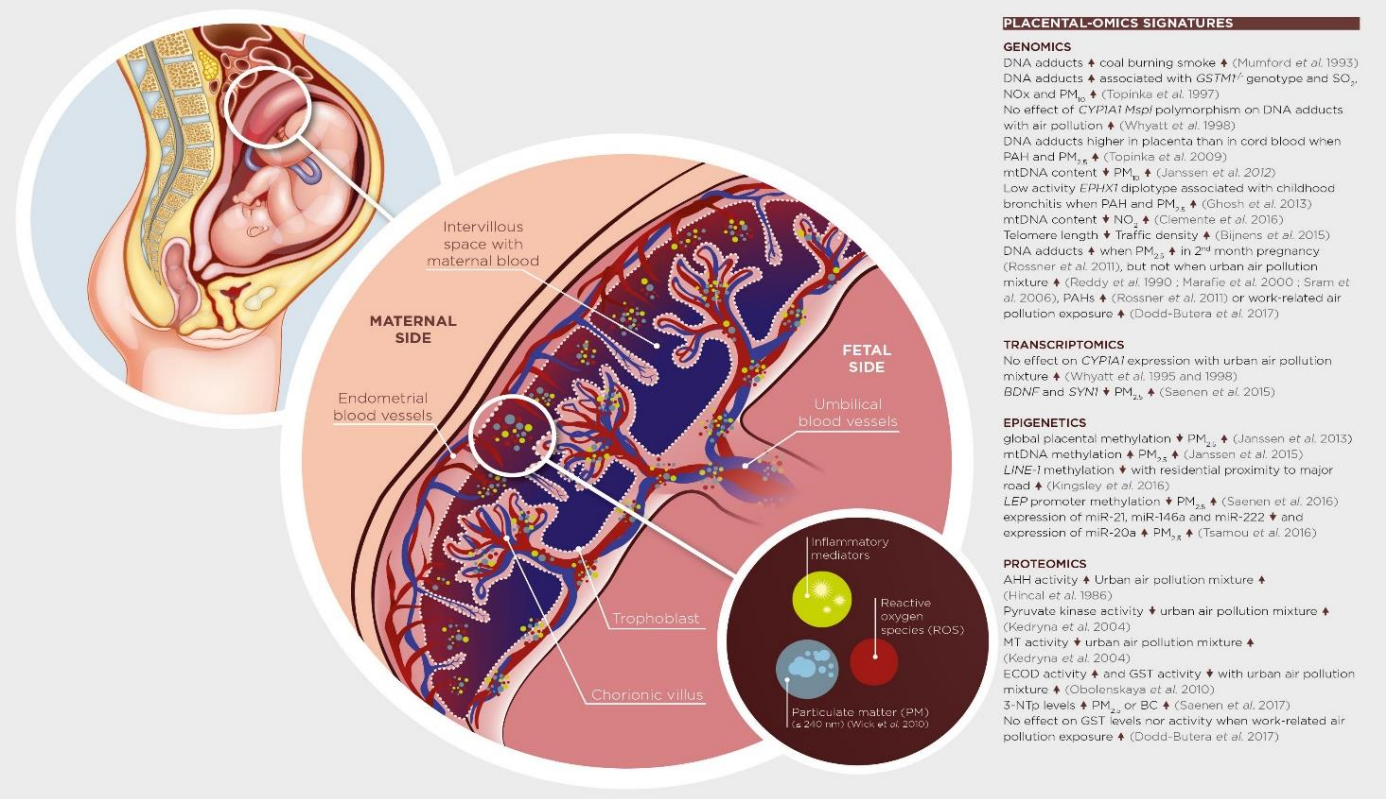


Figure 1. Placental migration of direct (particulate matter) and indirect (reactive oxygen species and inflammatory mediators) potential effectors of exposure to air pollution during pregnancy. The column on the right summarizes the -omics characteristics (genomics, transcriptomics, epigenetics, and proteomics) as described in this systematic review in association with exposure to *in utero* air pollution.

208

In this way, the placenta comes in contact with, contains and interacts with the substances to which both mother and fetus are exposed to during the timespan of the entire pregnancy. In addition, the placenta itself is an important endocrine organ regulating the production of hormones such as progesterone, human chorionic gonadotrophin (hCG), and human placental lactogen (hPL), to ensure the continuation of pregnancy and to acquire the appropriate maternal responses to optimize the development of the fetus.[38,39] Furthermore, within the feto-placental unit, a great number of signals are sent from the placenta to the fetus - and vice versa - to regulate developmental processes.[41] Such signals can also elicit the appropriate reactions to various environmental exposures. Together, all these properties make the placenta an essential organ for the regulation of fetal development. Indeed, placental dysfunction has been linked to for example the occurrence of preeclampsia and adverse birth outcomes such as intrauterine growth restriction.[42]

Intrauterine exposure to pollutants can lead to altered metabolic functions that may be detrimental for fetal development. For example, the embryonic brain has a great plasticity and its development depends on, and can be influenced by, various environmental factors.[325] The etiology of diseases in adulthood may have a fetal origin and may be attributed to the effects of adverse environmental exposures *in utero*. This causality concept is known as the Barker hypothesis or the Developmental Origins of Health and Disease (DOHaD). Professor David Barker was the first to recognize this potential link when he became concerned about the association between malnutrition during pregnancy and the development of coronary heart disease in adult life.[3] Since then, many implications of this hypothesis have been reported.[326–328] Adverse environmental exposures during pregnancy already identified in this context are active and passive cigarette smoke [6], and exposure to ambient air pollution [including nitrogen dioxide (NO_2) [7], polycyclic aromatic hydrocarbons (PAH) [8], and particulate matter (PM).[9] Particles with a diameter smaller than 500 nm are known to pass the placental barrier during the gestational period, while particles with a diameter smaller than 240 nm are even able to reach the fetal bloodstream (**Figure 1**), possibly affecting the newborn's metabolism before birth.[10]

APPENDIX A

Various reviews have already described the associations between prenatal ambient air pollution exposure and birth outcomes such as prematurity and birth weight.[272,329] However, none of these reviews described the placenta as an intermediate matrix having the potential to express distinct biological (-omics) signatures associated with prenatal exposure to ambient air pollution. Hence, the goal of this systematic review is to provide a structured overview and an evaluation of the current knowledge on the potential of placental tissue as a non-invasive biological matrix for the study of molecular -omics signatures that are associated with *in utero* exposure to ambient air pollution and are probably useful as early-life markers of disease development later in life. With this systematic review we aim to identify signatures in the -omics fields that already have been well addressed and those of which a substantial gap of knowledge still remains in the scope of epidemiological research involving the placenta as a tissue to identify sentinel biological effects of air pollution exposure during pregnancy.

MATERIALS AND METHODS

The goal of this systematic review was to provide an answer to the question: "Which -omics biomarkers have been analyzed in human placental tissue used as a non-invasive matrix in epidemiological research in association with prenatal exposure to air pollution in the context of disease development later in life?". The PECO elements that can be deduced from this question were used to determine the selection criteria to search and structure the articles for the synthesis of this review. These PECO elements are:

- "Population": human. In this article we focused on research conducted in an epidemiological context, thus not including research on human cell lines.
- "Exposure": ambient air pollution [including particulate matter with particles smaller than 2.5 µm ($PM_{2.5}$), particulate matter with particles smaller than 10 µm (PM_{10}), ultrafine particles, black carbon (BC), derivatives of nitrogen oxide (NOx), and polycyclic aromatic hydrocarbons (PAHs)]. We defined ambient air pollution as a mixture of indoor and outdoor pollutants, in both solid and gaseous form, and we excluded direct (maternal) or indirect (environmental) exposure to tobacco smoke from this concept.

- "Comparator": in this review were included both studies in which comparisons are made between groups exposed to either a higher or a lower concentration of air pollution, as well as studies with a continuous exposure scale.

- "Outcomes": placental -omics biomarkers and, if discussed, disease development or the development of adverse birth outcomes.

This systematic review was constructed according to existing guidelines on the structure of systematic reviews and maps.[330] An online database search was performed in January 2017, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (http://www.prisma-statement.org) to identify articles that are dealing with the scope of this review, without any limitation set on the publication date. Two investigators (LJL and NDS) were appointed to conduct the literature search, because of their expertise on the effects of air pollution in the placenta. These investigators read all papers, extracted, and archived the relevant information independently. The level of consensus between LJL and NDS was determined by performing a Cohen's kappa analysis. Any remaining discrepancies were resolved by consensus. The exploration was conducted on PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Scopus (http://www.scopus.com/), and ScienceDirect (http://www.sciencedirect.com). Only English MeSH-terms were used to form the search strings. First, a search was conducted with the key terms "placenta" and "air pollution". Next, additional searches were performed by replacing these terms with related search queries (for a list of all used queries see Supplemental Tables S1 and S2). Additionally, since we were interested in the link between -omics in the placenta and the development of disease, we replaced the air pollution-related MeSH-terms with the MeSH-terms "fetal origin adult disease", "barker hypothesis", "barker hypothesis fetal" and "barker hypothesis fetal origins" in the identification phase (**Supplemental Table S1 and S2**). Only primary research was included in this paper: in case a review article was found in the literature search, the list of references in this review was checked manually to determine if additional articles could be identified that met the inclusion criteria of this systematic review. If a full text could not be obtained, a request was sent via ResearchGate (https://www.researchgate.net/) or via the website of the journal

in which the article was published. In search for potential additional information from grey literature, we used a popular search engine (http://www.google.com), and accessed the OpenGrey (http://www.opengrey.eu), and Cochrane Library (http://onlinelibrary.wiley.com/cochranelibrary/) websites. First of all, we read the abstract of all papers that were found from the identification procedure and excluded the research articles on animals or human cell lines, since we wanted to put the emphasis solely on epidemiological research. The comparison of differences in placental -omics signatures between different (animal) models is beyond the scope of this systematic review. We also excluded comments on other research articles and the papers not written in English to avoid potential misinterpretation of the results due to incorrect translation. Subsequently, we examined the full text of the remaining articles and excluded those studying exclusively the effect of maternal active and/or passive smoking during pregnancy on placental -omics signatures or fetal health. These articles were excluded because air pollution is a complex mixture that takes into account the effects of various sources, such as traffic- and industry-related pollution, while research on smoking only focusses on the effects of tobacco use. Additionally, research articles that did not consider the measurement of -omics markers in the placenta were not included, because this review specifically focusses on the effects of air pollution exposure during pregnancy on the -omics biomolecular signatures of the placenta. For the remaining articles that were included in this systematic review, the content was examined in detail with a great focus on (i) the placental -omics marker(s) studied and the techniques used to measure them, (ii) the characteristics of prenatal exposure to ambient air pollution in association with the placental -omics marker(s), and (iii) whether the authors mentioned any association with disease development later in life. Finally, a descriptive analysis of these articles was made and a summary of the current knowledge has been provided based on the different -omics fields (genomics, epigenetics, transcriptomics, proteomics and metabolomics). In this way, existing gaps of knowledge in this research field could be established.

RESULTS

Using the initial MeSH-terms "placenta" and "air pollution", 118 articles could be identified (**Figure 2**). Replacing the MeSH-terms by alternative terms (**Supplemental Tables S1 and S2**), 268 additional records could be added to the list. No new articles were identified from reference lists of other reviews and no additional information could be retrieved from grey literature. From the total of 386 articles, 42 were excluded because they were not written in English. One study was excluded since it was a comment on another research article. The abstracts of the remaining articles were scanned for eligibility based on whether they pertained to epidemiological research. We excluded 70 animal studies and 25 studies using human cell lines.

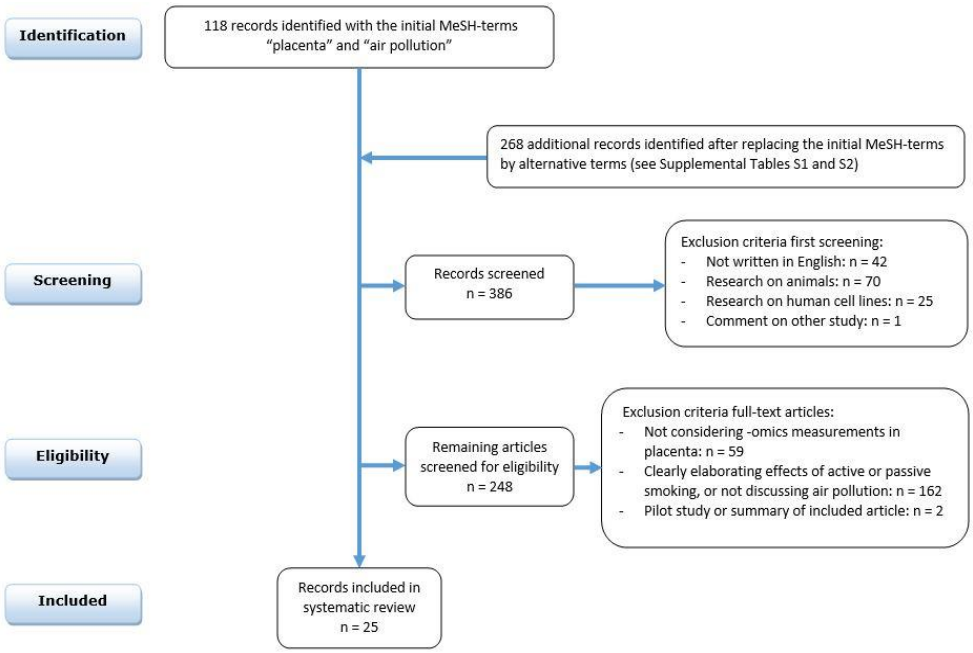


Figure 2. Flowchart of the selection protocol according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. From the 386 initially screened articles, 25 were included in this systematic review.

APPENDIX A

Of the remaining 248 articles, 59 were excluded because they did not report -omics measurements in human placental tissue and 162 were not included since the article only elaborated on the effects of active or passive maternal smoking and not on concomitant effects of exposure to ambient air pollution during the gestational period. One study of Topinka *et al.* [182] was considered a pilot study of one of the remaining articles of these authors [57], and one article of Sram *et al.* [331] summarized the latter study, so the results of these three studies were discussed simultaneously. The inter-rater variability as determined by the Cohen's kappa analysis was 0.98 (95% confidence interval: 0.96 – 0.99), which can be regarded as a value indicating an almost perfect agreement between LJL and NDS.

Twenty-five studies (**Supplemental Table S3 and Figure 1**) met all the selection criteria. The publication dates of the articles ranged from August 1990 to September 2016. Six articles discussed the triple relationship involving *in utero* air pollution exposure leading to molecular changes in placental tissue, with a direct or indirect descriptive link to adverse birth outcomes and/or the development of (chronic) diseases.[48,49,55,56,60,67] Five out of these six studies investigated a change in birth weight as an adverse outcome, three out of these five also looked at growth restriction [55,60,67], and one article studied prematurity of the neonate as an additional detrimental birth outcome.[56] Only one of these five studies investigated air pollution exposure during pregnancy, while looking at the associations with placental -omics markers and the development of a disease outcome later in life, namely childhood bronchitis.[49]

All 25 studies were observational, conducted in an epidemiological context, and used the placenta as a biological matrix to study molecular effects of prenatal ambient air pollution exposure. Among these studies, all categories of -omics markers were covered with exception of the placental metabolome and exposome. We identified eleven studies on the genome, five on the epigenome, two on the transcriptome, five on the proteome, one study with both genomic and transcriptomic topics, and one study covered topics on both genomics and proteomics (**Supplemental Figure 1**). The 25 included research articles showed a bottom-up approach for all -omics categories, focusing on specific preselected targets and their association with prenatal exposure to ambient air pollution.

Twelve of the 25 articles discussed the effects of PM air pollution on placental –omics (**Supplemental Figure 2**). More specifically, three studies investigated PM_{10} (one study in combination with other forms of air pollution, namely PAHs, SO_2 and NOx), while nine studies investigated $PM_{2.5}$. Exposure to $PM_{2.5}$ was often studied in combination with other air pollution components, such as PAHs (three studies) and black carbon (one study). Other forms of ambient air pollution were discussed separately as well, such as NOx (one study), and PAHs (one study). Seven articles analyzed a comparison of two groups of participants, based on their exposure to urban air pollution. Finally, four articles used proxies for air pollution exposure, such as the distance of the residence to a major road, residential traffic density, work-related air pollution exposure in maquiladoras (factories at the border between Mexico and the USA), and smoke from residential coal burning as a heating source.

DISCUSSION

Placental tissue in epidemiological research: advantages and disadvantages

All 25 studies that were selected for discussion in this review used placental tissue as a biological matrix for epidemiological research purposes. This temporary organ has the advantage that it can serve to evaluate biological outcomes of environmental exposures simultaneously in tissue with both maternal and fetal origin. Moreover, the sampling of placental tissue requires no invasive procedure, avoiding unnecessary potential damage to the fetus. The placenta shows to be a crucial tissue to study certain developmental processes, since it provides the necessary molecules for these mechanisms. In mice it has been shown that this organ produces serotonin at the earliest phases of pregnancy, which is an important factor in the development of the fetal central nervous system.[43] Five studies discussed in this review made a link between biomolecular characteristics of the placenta and health conditions that could interfere with human development later in life, more specifically a decrease in birth weight [48,55,56,67], fetal growth restriction [55,67] or the development of bronchitis in early childhood.[49] This shows that the placenta has the potential to serve as a tissue to study the link between

prenatal exposures and the effects on the (mal-)development of children in early life. Apart from the different functions of umbilical cord blood and the placenta during pregnancy, several molecular differences between both matrices have been identified such as different turnover rates of mitochondrial DNA (mtDNA).[44] In contrast to cord blood, which can encompass the effects of environmental exposures on the short term, the placenta can reflect the cumulative effect of prenatal exposures over the pregnancy period. In the context of the evaluation of exposure conditions on fetal development, biomolecular measurements in placental samples can be particularly useful since it has been suggested that changes in the placenta could be involved in the epigenetic regulation of fetal development, possibly to a slightly greater extent than in cord blood.[45]

A disadvantage of using placental tissue for research purposes is that obtaining representative sample aliquots is challenging as the placenta is composed of a heterogeneous mix of cells, blood vessels, chorionic villi, and membranes. Therefore, standardization of placental sampling is of great importance to account for the complexity of this tissue. Moreover, the sampling procedures carried out in several studies and cohorts using different protocols could introduce variability in the observed results and the conclusions drawn from this research. When comparing the sampling methods of the 25 studies included in this review, differences were identified in terms of sampling position on the placenta, the placental layers which were sampled, and the size of the tissue samples [ranging from 1 - 2 cm^3 [44] to 50 g].[69] In the context of relatively large numbers of samples or subjects under investigation in epidemiological studies and the related costs for molecular measurements, an additional disadvantage is that it is not always feasible to analyze multiple samples from the same placenta. Observational studies may consider pooling several biopsies of one placenta to further reduce sample variability. Suggestions for a more standardized protocol have already been made by Burton *et al.*, with regard to speed of sampling, aliquoting and preservation of the tissue to ensure sufficient quality of the DNA, RNA, and proteins for further analyses.[96] These authors advice to use a standardised grid to sample each placenta at minimal four different sites, take samples of 1 - 2 cm^3, and divide these biopsies into smaller aliquots according to

your -omics field of interest, and quickly snap freeze the samples after rinsing them in phosphate-buffered saline (PBS) at 4°C.[96]

Placental -omics signatures of prenatal air pollution exposure

At delivery, the placenta is a representative source of the morphological, functional, biological, and molecular information that has been accumulated during gestation. Therefore, it is a suitable matrix for postnatal investigation of potential associations between molecular (-omics) signatures and prenatal environmental influences. Several biomolecular characteristics related to diverse toxicological exposures have already been investigated in placental tissue. Not only direct DNA damage, but also changes in -omics (genomics, epigenetics, transcriptomics, proteomics, metabolomics and exposomics) signatures can occur due to hazardous environmental exposures such as ambient air pollution (**Table 1**). These alterations may possibly provide early effect predictors for human health risk due to *in utero* environmental exposures.[46] In this context, characteristic biomolecular signatures measured in humans may be considered biomarkers - which can be a chemical or its metabolite - biomolecules, or the product of an interaction between a substance and a target molecule or cell.[70] The measurement of placental -omics markers can provide useful insights on gestational exposure effects, susceptibility, and disease risk of the neonate.[46,71] Despite the fact that several changes in -omics fields have been characterized in placental tissue in association with air pollution exposure, two fields - metabolomics (discussed below) and exposomics - could not be sufficiently covered in the context of this systematic review because of the lack of studies on these topics.

APPENDIX A

Table 1. -Omics categories and placental markers analyzed in association with exposure to ambient air pollution during the gestational period

-Omics category	Placental markers
Genomics	- Telomere length [47] - Mitochondrial DNA content [44,48] - Presence of the low activity *EPHX1* (His/His) diplotype [49] - Presence of the *CYP1A1* MspI polymorphism [50] - DNA adduct levels [50–56,182]
Epigenetics	- Global DNA methylation level [59] - LINE-1 and AluYb8 DNA methylation levels [60] - Mitochondrial DNA methylation level [61] - *LEP* promoter methylation [62] - Levels of miR-21, miR-146a, miR-222, and miR-20a [63]

Transcriptomics	- Expression levels of
	  - *BDNF* [64]
	  - *SYN1* [64]
	  - *CYP1A1* [50,65]

Proteomics	- 3-NTp level [26]
	- Amount of metallothionein [66]
	- GST level [51]
	- Activity of
	  - AHH [67]
	  - Pyruvate kinase [68]
	  - GST [51,69]
	  - ECOD [69]

Metabolomics	/

Abbreviations: 3-NTp, 3-nitrotyrosine; AHH, Aryl hydrocarbon hydroxylase; BDNF, Brain-derived neurotrophic factor; CYP1A1, Cytochrome (CYP) P450 1A1; ECOD, 7-ethoxycoumarin O-deethylase; EPHX1, Epoxide hydrolase 1; GST, Glutathione S-transferase; His, Histidine; LEP, Leptin; miR, MicroRNA; SYN1, Synapsin 1

The field of exposomics encompasses all the environmental exposures for an organism during its lifetime.[332] Placental exposomics have for example been studied in mothers known to be obese or diabetic at the moment of gestation.[333] In case of investigating the effects of *in utero* exposure to ambient air pollution one study can be cited which measured asbestos fibers as a part of the exposome in the placentas of stillborn babies.[334] Several intermediate markers including telomere length and microRNA (miRNA) expression patterns have been studied as a proxy-effect of ambient air pollution exposure on exposomics.[35,335] However, the full placental exposome regarding environmental air pollution exposure is a complex entity of which the parts still need to be assembled.

Genomics (Table 2)

Direct DNA damage and damage through DNA adducts were two of the first placental markers used to evaluate the health significance of genomic insults through prenatal ambient air pollution exposure. As early as 1990, 32P-postlabeling was performed in placental tissue to study the extent of DNA damage that could be inflicted by exposure to PAHs during pregnancy.[54] Ten years later, a similar study was published on DNA adducts in placental samples using two different techniques, i.e. nuclease P1 and butanol extraction enhancement prior to 32P-postlabeling.[52] Both studies came to the same conclusion: the levels of placental DNA adducts did not differ significantly between women exposed to airborne PAHs by either residential wood combustion [54] or pollution from oil well fires [52] compared with non-exposed women. In a recent study, lack of association was also found between placental PAH-adducts and exposure to work-related air pollution at the US-Mexican border.[51] Mumford *et al.* came to the opposite conclusion in a study on placental DNA-adduct levels and PAH exposure during pregnancy: the adduct levels increased when mothers were exposed to smoke of coal burning during pregnancy, however, these results lack statistical confirmation.[53] Furthermore, a study on ambient $PM_{2.5}$ and PM_{10} air pollution also did not show an association between placental DNA-adduct levels and exposure to air pollution.[56] Hence, the consistent negative results from these independent studies may point to a molecular effect other than the formation of DNA adducts

in the placenta associated with maternal air pollution exposure. Topinka *et al.* compared placental adduct levels with those in cord blood following *in utero* exposure to PAHs and $PM_{2.5}$, and showed that the total level of DNA adducts was significantly higher in cord blood compared to placenta.[58] Other studies in cord blood also showed positive relationships between DNA adduct levels and exposure to air pollution [183], which indicates that these hazardous airborne substances could affect DNA adduct levels in other tissues than the placenta.

Fetuses are able to adapt their mitochondrial structure and metabolism when the supply of nutrients is limited or compromised. Mitochondria are the biochemical power plants of cells providing energy through the production of adenosine-5'-triphosphate (ATP) via oxidative phosphorylation. These intracellular organelles contain multiple copies of circular DNA - mitochondrial DNA (mtDNA) - of approximately 16 kb in length which are vulnerable to reactive oxygen species (ROS) because of close proximity to the electron transport chain and inefficient DNA repair capacity.[336] The estimated mutation rate of mtDNA is 5 - 15 times higher compared to nuclear DNA.[337] Changes in placental mtDNA content may represent a biological effect along the path linking air pollution to adverse effects on the unborn. In placental tissue of 174 mother-newborn pairs of the Belgian birth cohort ENVIR*ON*AGE, an inverse association was found between third trimester PM_{10} (and NO_2) exposure and placental mtDNA content (-17.4%, 95% CI: -31.8 to -0.1%, for an increment of 10 $\mu g/m^3$ in PM_{10} exposure; $p = 0.05$).[44] A similar inverse association was found in the Spanish INMA birth cohort between placental mtDNA content and gestational exposure to traffic-related NO_2 air pollution (-4.9%, 95% CI: -7.9 to -1.8% for an increment of 10 $\mu g/m^3$ in NO_2 exposure; $p = 0.003$).[48] The discrepancy in effect-size can be explained by the very dynamic nature of placental mtDNA.

It is known that mtDNA fluctuates under the influence of age, ethnicity, and tissue investigated, but most importantly depends on oxidative stress level, cellular antioxidant capacity, type of environmental factor, and dose of exposure.[338,339] Further research on this topic is essential, since alterations in placental mitochondrial function or capacity of the placenta may influence fetal energy provision and development.[340]

APPENDIX A

Telomere length predicts life span early in life and captures the history of inflammatory and oxidative stress effects of exposure to environmental stressors.[35,341] For example, exposure-related oxidative stress and inflammation are known to contribute to telomere shortening.[342] Bijnens *et al.* investigated changes in placental telomere length in twins in correlation with traffic-related exposure.[47] In this study, three indicators of exposure were assessed, i.e. the distance from the residential address of the mother to the nearest major road, traffic density within a 200 m buffer from the residence, and residential greenness. The authors concluded that placental telomere length was longer in association with a doubling of the residential distance to a major road (5.3%, 95% CI: 1.9 to 8.9%; p = 0.003), and shorter with a doubling in traffic density (-4.0%, 95% CI: −7.6 to −0.2%; p = 0.04).

Other genomic factors of susceptibility in the context of health and disease are DNA polymorphisms. Specific polymorphisms can cause an alteration in the metabolic capacity of cells as to the degradation and/or elimination of toxic substances, such as particle-bound chemicals derived from tobacco smoke or ambient air pollution. In turn, these metabolic changes could entail a new risk of disease development.[343] Research has focused specifically on the associations between *in utero* exposure to air pollution and placental genotypes related to detoxification mechanisms. One of the most important actors in this process is cytochrome P450 1A1 (CYP1A1), which is expressed in various tissue types throughout the body including the placenta, and fulfils both a detoxifying and a bioactivating role. This enzyme can bioactivate pro-carcinogenic substances such as PAHs to form adducts with DNA in tissues of both the mother and child during pregnancy.[344] Whyatt *et al.* focused on gestational air pollution exposure and its effects on changes of placental *CYP1A1*.[50] In the genomic category, the authors investigated the association between the homozygous (*Mspl*+/+) or heterozygous (*Mspl*+/-) presence of the *CYP1A1* Mspl polymorphism in placental tissue between smokers and non-smokers within areas heavily or less polluted with PAHs. An association between the placental presence of this polymorphism and the formation of DNA adducts due to PAH air pollution could not be demonstrated.[50] In addition, Sram et al. studied the association between *CYP1A1* polymorphisms and PAH levels in association with birth weight, but a significant effect of these

placental polymorphisms on birth weight following maternal PAH exposure was not found.[56] $PM_{2.5}$ and PM_{10} levels were also measured in this study, but no effects of these air pollution components were mentioned. Glutathione S-transferase M1 (GSTM1) and N-acetyl transferase 2 (NAT2) are two other enzymes involved in the detoxification system of cells. Studies conducted on the genotypes of these two enzymes in human placental tissue showed that both genes interact with ROS, but only the null phenotype *GSTM1-/-*, unlike *GSTM1+/-* or *GSTM1+/+,* was correlated with maternal exposure to SO_2, NOx and PM_{10} during pregnancy.[57,182] The placenta did not only prove to be a useful tissue for genomic analyses of GST polymorphisms in connection with air pollution exposure, but also for studying the proteomic level of placental GST activity (see proteomics section).[69]

Table 2. Studies describing the associations between prenatal ambient air pollution exposure and changes in placental genomic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Reddy *et al.* (1990)	4 non-smoking women exposed to wood smoke during pregnancy and 5 non-exposed controls from Massachusetts	Urban air pollution	No significant differences in DNA-adduct levels between exposed and non-exposed mothers.
Mumford *et al.* (1993)	38 placental samples from Xuan Wei (China) exposed to coal combustion smoke during pregnancy and 19 samples from controls living in Beijng, using natural gas as heating source.	Smoke from coal combustion	Total DNA-adducts detected in 52% of placentas of exposure group compared to 5.3% of the samples of the control group (no statistics performed)
Topinka *et al.* (1997)	158 mothers (113 non-smokers and 45 smokers) in two districts of the Czech Republic with different exposure levels of air pollution	Average monthly concentrations of SO_2, NOx, PAH and PM_{10} from January 1994 until January 1995	Increased levels of DNA-adducts in samples of the highly exposed regions compared to the lower exposed regions in placentas with the *GSTM1* null genotype ($p = 0.029$) No effect of NAT2 genotype on DNA adduct levels in correlation with air pollution exposure
Whyatt *et al.* (1998)	70 subjects from Krakow with higher levels of air pollution and 90 subjects from Limanowa, a less polluted city in Poland	Average annual concentration of 37 µg/m³ of ambient respirable particles in least exposed group and 78 µg/m³ in the most exposed group, in the year prior to delivery (particle size not defined)	No significant associations between PAH-adduct levels, presence of the *CYP1A1 MspI* polymorphism and exposure to air pollution.

Continued

Table 2. **Continued**

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Marafie *et al.* (2000)	40 mothers exposed to oil well fires and 180 non-exposed Kuwaiti mothers	Urban air pollution	No significantly different levels of DNA-adducts between mothers of different exposure groups
Sram *et al.* (2006)	199 subjects born in 1994-1995 for DNA-adduct analyses and 1013 subjects born in 2000-2002 for genotyping. Samples collected in two districts of the Czech Republic with different levels of air pollution	Urban air pollution	No significant associations between placental DNA-adduct levels and birth weight, and no effects of air pollution on birth weight or DNA-adduct levels identified.
Topinka *et al.* (2009)	Placentas from 79 individuals born in 2007 and 2008 in Prague (Czech Republic)	B[a]P, PAHs and $PM_{2.5}$ levels (no mean values provided)	Total DNA-adduct levels are significantly higher in cord blood compared to placental tissue ($p < 0.001$)
Rossner *et al.* (2011)	891 subjects born between 1994 and 1999 in two districts of the Czech Republic with either high or low levels of air pollution exposure	Average concentrations of PAHs and $PM_{2.5}$ for each month of pregnancy	No significant associations between 8-oxodG-adduct levels and PAH levels, but a significant increase in 8-oxodG-adduct levels with increased $PM_{2.5}$ exposure in second month of pregnancy (OR = 1.68, 95% CI: 1.28 to 2.19; $p < 0.001$)
Janssen *et al.* (2012)	174 individuals from the ENVIR*ON*AGE cohort (Belgium)	10 µg/m³ increase in PM_{10} (22.7 ± 3.7 µg/m³ for entire pregnancy)	16.1% decrease in mtDNA content in association with exposure during the last month of pregnancy (95% CI: −25.2 to −6.0%, $p = 0.003$)

Continued

Table 2. **Continued**

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Ghosh *et al.* (2013)	n = 793 randomly selected from children born between 1994 and 1998 in two districts of the Czech Republic	100 ng/m³ increase in PAH (63.4 ng/m³ ± 51.5 ng/m³) and a 25 µg/m³ increase in $PM_{2.5}$ (22.8 µg/m³ ± 11.9 µg/m³) for the entire pregnancy period	Significantly higher effect of both PAH (OR = 1.5, (95% CI: 1.2 to 1.9) and $PM_{2.5}$ (OR = 1.5) exposure on the development of childhood bronchitis, associated with the low activity *EPHX1* (His/His) diplotype
Bijnens *et al.* (2015)	n = 211 twins of the East Flanders Prospective Twin Study (Belgium)	Doubling of the residential distance to a major road and doubling in traffic density, as proxy for maternal traffic/air pollution exposure	5.3% increase of RTL with every doubling of the residential distance to a major road (95% CI: 1.9 to 8.9%; p=0.003) and a decrease in RTL with 4.0% for every doubling in traffic density (95% CI: −7.6 to −0.2, p = 0.04)
Clemente *et al.* (2016)	n = 376 (INMA cohort, Spain) and n = 550 (ENVIR*ON*AGE cohort, Belgium)	10 µg/m³ increase in NO_2 (25.5 ± 11.4 µg/m³ in INMA cohort and 21.1 ± 4.2 µg/m³ in ENVIR*ON*AGE cohort respectively for the entire pregnancy period)	1) ENVIR*ON*AGE cohort: 11.1% decrease in mtDNA for the second trimester (95% CI: -19.9 to -1.24%; p = 0.03) and 13.5% decrease in mtDNA for the third trimester of pregnancy (95% CI: -20.1 to -6.4%; p = 0.003). 2) INMA cohort: decrease in mtDNA content for the first (-4.1%, 95% CI: -7.1 to -1.1%; p = 0.007), second (-5.0%, 95% CI: -8.0 to -2.0%; p = 0.002) and third (-4.9%, 95% CI: -7.9 to -1.8%; p = 0.003) trimester, and for the entire pregnancy (-5.5 %, 95% CI: -8.8 to -2.1%, p = 0.002)

Continued

Table 2. Continued

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Dodd-Butera *et al.* (2016)	n = 54 from Tijuana, Mexico	Work-related PAH air pollution exposure from working in maquiladora factories	No significant differences in DNA-adduct levels

Abbreviations: 8-oxodG, (8-oxo-2'-deoxyguanosine); B[a]P, Benzo[a]pyrene; CI, Confidence interval; EPHX1, Epoxide hydrolase 1; GSTM1, Glutathione S-transferase M1; mtDNA, Mitochondrial DNA; NAT2, N-acetyl transferase 2; NO_2, Nitrogen dioxide; NOx, Nitrogen oxides; OR, Odds ratio; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; PM_{10}, Particulate matter with a diameter smaller than 10 μm; RTL, Relative telomere length; SO_2, Sulfur dioxide.

Epigenetics (Table 3)

The most commonly characterized epigenetic marking process is DNA methylation, which involves the addition of a methyl group to the carbon-5 position of cytosine residues of the dinucleotide CpG. DNA methylation undergoes critical modification during early *in utero* life. After fertilization and prior to implantation, DNA methylation patterns are largely erased but are re-established by *de novo* DNA methyltransferases (DNMTs) in the blastocyst stage.[345] These waves of epigenetic reprogramming likely make early embryonic development a critical period during which nutritional, environmental, and metabolic factors affect the developmental establishment of epigenetic regulation.[346] The placenta exhibits a different methylation profile compared to fetal somatic tissue which is probably needed to generate cells with a broad developmental potential and the correct initiation of embryonic gene expression.[347] Indeed, the placenta shows considerable developmental plasticity which is important for adaptation to fetal and maternal signals including hormonal and environmental exposures or other responses to *in utero* conditions.[348] Hence, the placenta contains information on DNA methylation patterns revealing the environmental impact to which the fetus has been exposed during gestation.

An expanding body of evidence suggests that exposures to hazardous environmental factors are important determinants for altered DNA methylation-related programming during early life. These alterations can persist throughout the course of life, thereby leading to pathological conditions in adulthood. Recently, Vaiserman summarized clinical and epidemiological evidence in support of epigenetic factors that may mediate the link between early-life exposures and long-term health outcomes.[349] Changes in DNA methylation patterns of placental tissue have been disclosed in association with adverse maternal exposures such as alcohol and tobacco smoke [350], however only recently placental epigenetic signatures have been identified in association with exposure to ambient air pollution. Janssen *et al.* were the first to investigate the association between $PM_{2.5}$ exposure during pregnancy and the global DNA methylation levels in placental tissue.[59] For the entire pregnancy period they found that an increase of 5 μg/m³ in $PM_{2.5}$ exposure correlated with a relative decrease of 2.2% in global placental DNA methylation (95% CI: -3.7 to -0.7%; p = 0.004). These findings have been

replicated by Kingsley *et al*.[60] The authors showed that pregnant mothers living closer to major roads, as a marker of traffic-related air pollution, had lower levels of placental DNA methylation in LINE-1 (-0.82%, 95% CI: −1.57 to −0.07; p = 0.03) but not AluYb8 repetitive elements, which can be regarded as surrogate markers of global DNA methylation.

Another interesting finding of the study of Janssen *et al.* was that the early gestational stage from fertilization up to and including implantation - a critical period for methylation reprogramming - is likely to represent a highly sensitive window for the effects of $PM_{2.5}$ exposure on placental DNA methylation as measured at birth.[59] The health implications of these findings should be further investigated, since it has been shown that overall hypomethylation patterns in the placenta could be an indication of an increased risk to birth defects such as spina bifida.[351] Furthermore, associations have been found between hypomethylation of specific promoters and adverse birth conditions such as low birth weight.[352]

Recently, attention has been drawn to the methylation pattern of a specific gene in the placenta, namely the promoter region of the leptin (*LEP*) gene. Leptin is an important hormone during pregnancy, since it plays a crucial role in fetal growth and development through its function in energy metabolism.[353] An interquartile range increment (IQR) of $PM_{2.5}$ exposure (7.5 µg/m³) was associated with a 1.4% decrease in placental methylation of the *LEP* promoter region (95 % CI: -2.7 to -0.2%; p = 0.02).[62] In previous research, a decrease in *LEP* methylation has been associated with gestational syndromes such as pre-eclampsia and impaired glucose tolerance.[354,355] The intricate connection between *LEP* methylation, PM exposure, and disease phenotype should be explored more in depth by studying potential ailments in childhood that may arise from these placental changes.

Besides the nuclear genome, the mitochondrial genome can undergo epigenetic modifications as well. For example, maternal emotional stress during pregnancy has shown to alter gene expression patterns in placental mitochondria, which can eventually affect the temperamental development of the child in early life.[356] DNA methylation in specific regions of the mitochondrial genome has been shown to substantially mediate the association between $PM_{2.5}$ exposure during gestation and placental mtDNA content which could reflect signs of mitophagy and

mitochondrial death.[61] However, the epigenetic changes in mtDNA patterns linked to air pollution exposure have not yet been studied in the context of developmental outcomes of the newborn. Therefore, further exploration of mitochondrial gene expression regulation by DNA methylation is of paramount importance to unravel these potentially important relationships.

A type of epigenetic mark that has not yet been investigated to a great extent in the context of prenatal air pollution exposure is microRNA (miRNA).[335] MiRNAs are endogenous, single-stranded, short non-coding RNA sequences (approximately 22 nucleotides) that regulate gene expression at the post-transcriptional level. Different cell types have both common and unique miRNA expression patterns, which can be influenced by developmental and pathologic states. The human placenta expresses a distinct subset of miRNAs, but although the functions of these placental epigenetic marks are largely unknown, recent research has revealed a functional role for miRNAs in placental biology.[357] The presence of placental miRNAs in the maternal circulation is interesting as it could lead to the discovery of biomarkers of placental dysfunction or pregnancy-related disease.[358] Only one study has described changes in placental miRNA expression in association with prenatal air pollution exposure. A relative decrease in the placental expression of miR-21 (-33.7%, 95% CI: -53.2 to -6.2%; p = 0.022), miR-146a (-30.9%, 95% CI: -48.0 to -8.1%; p = 0.012), and miR-222 (-25.4%, 95% CI: -43.0 to -2.4%; p = 0.034) was found in association with an increase of 5 μg/m³ $PM_{2.5}$ during the second trimester of pregnancy, whereas a positive association was described between first trimester $PM_{2.5}$ air pollution exposure and the expression of placental miR-20a (+70.9%, 95% CI: 16.7 to 150.3%; p = 0.007) and miR-21 (+73.7%, 95% CI: 11.7 to 170.1%; p = 0.015).[63] A common target of these miRNAs is the tumor suppressor phosphatase and tensin homolog (PTEN) which also showed an altered expression in association with $PM_{2.5}$ exposure (+59.6% per 5 μg/m³ increment, 95% CI: 26.9 to 100.7%; p < 0.0001) and, as expected, an inverse correlation with the levels of these miRNAs, since increasing levels of miRNAs are known to block the expression of their associated targets.[63]

Table 3. Studies describing the associations between prenatal ambient air pollution exposure and changes in placental epigenetic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Janssen *et al.* (2013)	240 samples from the ENVIR*ON*AGE birth cohort (Belgium)	5 μg/m³ $PM_{2.5}$ increase (17.4 ± 3.6 μg/m³ for entire pregnancy)	Decrease in global DNA methylation for whole pregnancy (-2.2%, 95% CI: -3.7 to -0.7%; p = 0.004), first trimester (-2.4%, 95% CI: -3.6 to -1.2%; p = 0.0001) and second trimester of pregnancy (-1.5%, 95% CI: -2.7 to -0.4%; p = 0.01)
Janssen *et al.* (2015)	381 mother-newborn pairs from the ENVIR*ON*AGE birth cohort (Belgium)	3 μg/m³ (IQR) increase in $PM_{2.5}$ (16.7 ± 2.3 μg/m³)	Increased mtDNA methylation levels (0.5%, 95 % CI: 0.2 to 2.2%; p < 0.05) and decrease of mtDNA content with 15.6% (95% CI: -23.9 to -6.4%; p < 0.05)
Kingsley *et al.* (2016)	471 mother-infant pairs from the RICHS cohort (Rhode Island, USA)	Proximity of the residential distance to a major road as proxy for air pollution exposure	0.82% decrease in mean LINE-1 methylation levels (95% CI: −1.57 to −0.07; p = 0.03)

Continued

Table 3. Continued

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Tsamou *et al.* (2016)	210 mother-child pairs from the ENVIR*ON*AGE cohort (Belgium)	5 µg/m³ increase in $PM_{2.5}$ (16.38 ± 5.29 µg/m³ for the first trimester of pregnancy and 16.74 ± 5.82 µg/m³ for the second trimester of pregnancy)	Decreased expression of miR-21 (-33.7%, 95% CI: -53.2 to -6.2%; p = 0.02), miR-146a (-30.9%, 95% CI: -48.0 to -8.1%; p = 0.012) and miR-222 (-25.4%, 95% CI: -43.0 to -2.4%; p = 0.034) for the second trimester of pregnancy and an increased expression of miR-20a (+70.9%, 95% CI: 16.7 to 150.3%; p = 0.007) and miR-21 (+73.7%, 95% CI: 11.7 to 170.1%; p = 0.015) in the first trimester.
Saenen *et al.* (2017)	361 samples from the ENVIR*ON*AGE birth cohort (Belgium)	7.5 µg/m³ (IQR) increase in $PM_{2.5}$ (15.5 ± 4.9 µg/m³ for the second trimester)	A 1.4% decrease in *LEP* promoter methylation for the second trimester of pregnancy (95% CI: -2.7 to -0.2%; p = 0.02)

Abbreviations: CI, Confidence interval; IQR, Interquartile range; *LEP*, Leptin; miR, Micro RNA; mtDNA, Mitochondrial DNA; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm

APPENDIX A

Transcriptomics (Table 4)

The area of placental transcriptomics has been poorly addressed in association with exposure to air pollution during pregnancy. One possible explanation for this is the difficulty to obtain placental tissue aliquots with a sufficiently high RNA quality for whole transcriptome analyses, since RNA starts degrading quickly after sampling.[359] However, various reviews have discussed the transcriptome of the placenta in the context of negative birth outcomes.[360,361] Recent research has shown that the placenta contains several distinct gene expression patterns compared with other tissues in the human body, for example when the number of slice variants and the expression levels of regulators involved in splicing are concerned.[362] Quantitative PCR, micro-array analysis, and RNA sequencing have proven to be indispensable for the analysis of exposure effects on transcriptomic alterations, potentially leading to perturbation of developmental and biological mechanisms. Apart from their work in the genomic field, Whyatt *et al.* also investigated the effects of prenatal exposure to air pollution on the placental expression of *CYP1A1*.[50,65] However, in both studies significant results concerning this gene could not be established, which was in accordance with the absence of an association in the genomic field of their research on *CYP1A1* (see section 4.2.1. Genomics).

Saenen *et al.* investigated the placental expression levels of ten genes in the *brain-derived neurotrophic factor (BDNF)* pathway in connection with PM air pollution.[64] A 5 µg/m³ increase in residential $PM_{2.5}$ exposure of the mother during the first trimester of pregnancy was associated with a 15.9% decrease in expression of placental *BDNF* (95% CI: −28.7 to −3.2%, p = 0.015), and with a 24.3% decrease in *synapsin 1 (SYN1)* expression (95% CI: −42.8 to −5.8%, p = 0.011) which is affected by the actions of *BDNF*. Proper functioning of this pathway in the placenta is crucial for normal fetal development, since altered BDNF expression in this tissue has been associated with negative birth outcomes such as fetal growth restriction.[363]

Table 4. Studies describing the associations between prenatal ambient air pollution exposure and changes in placental transcriptomic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Whyatt *et al.* (1995)	70 subjects from a city with higher levels of air pollution (Krakow) and 90 subjects from a less polluted city (Limanowa) in Poland	Average of 80 µg/m³ PM_{10} in highly polluted area (range, 23.4-154.2 µg/m³), no data available for Limanowa	No significant difference in *CYP1A1* mRNA levels between low and high polluted area (r = -0.4; p = 0.14).
Whyatt *et al.* (1998)	70 subjects from Krakow with higher levels of air pollution and 90 subjects from Limanowa, a less polluted city in Poland	Average annual concentration of 37 µg/m³ of ambient respirable particles in least exposed group and 78 µg/m³ in the most exposed group, in the year prior to delivery (particle size not defined)	PAH-adduct levels not significantly associated with *CYP1A1* mRNA (r = –0.10; p = 0.2)
Saenen *et al.* (2015)	90 randomly selected mother-child pairs from the ENVIR*ON*AGE birth cohort (Belgium)	5 µg/m³ increase in $PM_{2.5}$ (15.4 ± 5.4 µg/m³ for the first trimester, 17.6 ± 7.0 µg/m³ for the second trimester and 18.7 ± 6.0 µg/m³ for the third trimester of pregnancy)	15.9% decrease in placental *BDNF* expression (95% CI: –28.7 to –3.2%; p = 0.015) and a 24.3% lower expression of *SYN1* (95% CI: –42.8 to –5.8%, p = 0.011)

Abbreviations: *BDNF,* Brain-derived neurotrophic factor; CI, Confidence interval; *CYP1A1,* Cytochrome (CYP) P450 1A1; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 µm; PM_{10}, Particulate matter with a diameter smaller than 10 µm, *SYN1,* Synapsin 1.

APPENDIX A

Proteomics (Table 5)

Although proteins are the end products of the transcription of genomic sequences, there is no linear relationship between a genome and its resulting proteome because of alternative splicing and the production of non-functional proteins.[72] Therefore, it is essential that proteomics signatures are studied as a separate -omics field which can complement the finding in other -omics categories.[72] Proteomic characteristics have been investigated in placental tissue in association with maternal tobacco smoking habits and other exposures to toxicants during pregnancy.[364] Considerable focus has been put on proteins that play an essential role in the detoxification system of the cells. The activity of placental aryl hydrocarbon hydroxylase (AHH), which is the most important metabolizer of PAHs, was significantly higher in placentas obtained from mothers who lived in an environment exposed to urban air pollution compared with the control group.[67] Two other cellular detoxification indicators, glutathione S-transferase (GST) and 7-ethoxycoumarin O-deethylase (ECOD), showed opposite associations with increasing air pollution levels: ECOD activity significantly increased with increasing ambient air pollution (related to industry and traffic-exhaust), while the GST activity decreased under the same conditions.[69] Work-related exposure to another source of PAH air pollution during pregnancy, such as in the "maquiladoras" at the US-Mexican border, had no effect on the placental GST level or activity.[51] Detoxification processes are important for normal cellular functioning, but the maintenance of the delicate redox balance of the cell is a crucial factor as well. In controlling oxidative stress, metallothionein (MT) is an important protein for fixation and transport of metals. In a study of Sorkun *et al*., a significant increase in the amount of placental MT was observed in regions with higher levels of air pollution exposure.[66]

Another area of interest in placental proteomics addresses the energy system of the cell. Pyruvate kinase, an essential enzyme in the process of glycolysis, showed a significant increase of activity in placental tissue of women who lived in more polluted areas.[68] The level of this protein was also increased in placental tissue of preeclampsia pregnancies, a condition characterized by excessive inflammatory reactions in the placenta.[365] This parallels prenatal exposure to air pollution, as inflammation is the most likely mode of action triggered by ambient air pollution.[44]

Because glycolysis is a very important metabolic pathway of energy production in the placenta, more attention is needed as to the effects of air pollution on this critical pathway during gestation.[366]

Not only the intact proteins themselves, but also the products of protein degradation or modification can be measured as biomarkers of placental damage caused by detrimental influences during pregnancy. Protein damage can be caused by processes such as oxidative stress and inflammation. In this context, the tyrosine groups of proteins can be modified into 3-nitrotyrosine (3-NTp) by peroxinitrite, which is an intermediate of oxidative or nitrosative stress. Recently, a positive association was found between placental 3-NTp levels and both $PM_{2.5}$ exposure (+35.0% for a 3.5 μg/cm³ increment in $PM_{2.5}$, 95% CI: 13.9 to 60.0%; $p < 0.0006$) and BC exposure (+13.9% for a 0.36 μg/m³ increment in BC, 95% CI: −0.2 to 29.9%; $p = 0.05$), which is in line with recent studies on mice.[26] These animals showed increased placental 3-NTp levels which correlated with exposure to air pollution-related diesel exhaust.[75]

Table 5. Studies describing the associations between prenatal ambient air pollution exposure and changes in placental proteomic markers

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Hincal (1986)	152 mother-child pairs from residential Ankara and 125 mother-child pairs from the more rural areas surrounding Ankara	Urban air pollution	AHH activity was significantly higher in placental tissue of women living in Ankara compared to women of the rural areas ($p < 0.001$)
Kedryna *et al.* (2004)	15 women from Chorzow and Krakow (polluted areas) and 8 women from the Bieszczady Mountains (less polluted area) in Poland	Urban air pollution	Significant decrease in pyruvate kinase activity in more polluted areas ($p < 0.001$)
Sorkun *et al.* (2007)	Samples of 92 mothers: 33 smokers, 29 exposed to air pollution and 30 non-smokers residential in a rural area with lower levels of air pollution	Urban air pollution	Higher levels of metallothionein in group exposed to air pollution, compared to mothers living in rural area ($p = 0.013$)
Obolenskaya *et al.* (2010)	143 mothers who gave birth between 1991-1999 in polluted areas of Ukraine and Belarus, and a less polluted area in the east of Poland.	Urban air pollution	Significantly lower GST activity ($r_s = -0.27$; $p = 0.05$) and higher ECOD activity ($p < 0.05$) in highly polluted areas compared to the lower polluted areas

Continued

Table 5. Continued

Author	Study population	Increase in analyzed air pollution component (average ± standard deviation if available)	Effect on placental -omics marker
Saenen *et al.* (2016)	330 mother-child pairs from the ENVIR*ON*AGE birth cohort (Belgium)	3.5 μg/m³ (IQR) increase in $PM_{2.5}$ (16.1 ± 2.4 μg/m³ for whole pregnancy) and 0.36 μg/m³ (IQR) increase in BC (0.97 ± 0.28 μg/m³ for entire pregnancy)	35.0% increase in 3-NTp levels for increased $PM_{2.5}$ levels (95% CI: 13.9 to 60.0%; p = 0.0006) and 13.9% increase in 3-NTp levels for increased BC levels (95% CI: −0.2 to 29.9%; p = 0.05) during the entire pregnancy period
Dodd-Butera *et al.* (2016)	n = 54 from Tijuana, Mexico	Work-related PAH air pollution exposure from working in maquiladora factories	No significant difference in GST level (p = 0.243) or GST activity (p = 0.965) between women working in maquiladoras and women from non-exposed area

Abbreviations: 3-NTp, 3-nitrotyrosine; AHH, Aryl hydrocarbon hydroxylase; BC, Black carbon; CI, Confidence interval; CYP1A1(*2A), Cytochrome (CYP) P450 1A1 (2A); ECOD, 7-ethoxycoumarin O-deethylase; EPHX1, Epoxide hydrolase 1; GST, Glutathione S-transferase; IQR, Interquartile range; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm.

APPENDIX A

Metabolomics

Metabolomics is a research area that deals with changes in small metabolites (lipids, amino acids, sugars, etc.) as a consequence of altered metabolism by internal or external influences.[367] This -omics study area has been broadly addressed within several research topics concerning placental tissue. The placental metabolome of complicated pregnancies has been compared with that of normal pregnancies to investigate the molecules associated with adverse outcomes such as neural tube defects.[368] However, the placental metabolome characteristic of prenatal exposure to ambient air pollution did not deserve any attention until now. Metabolomic parameters linked to the effects of air pollution have been studied to a small extent in other human matrices such as umbilical cord blood plasma (e.g. oxylipins) and lung lavage fluid.[369,370] Since these results showed specific metabolic signatures associated with air pollution exposure, this should be an incentive to further investigate tissues such as the placenta to reveal early-life changes in metabolic pathways due to adverse exposures during pregnancy.

Triple relationship between exposure to ambient air pollution, placental biomarkers and disease development

As stated by Professor David Barker in the early 1990s, the occurrence of diseases later in life may already be initiated during fetal development as a result of detrimental *in utero* exposures and direct or indirect influences of placental involvement.[3] To fully comprehend the complexity of the fetal origin of disease, it is crucial to investigate the intricate triple relationship between exposure, molecular effect and clinical outcome (**Table 6**). In earlier research, morphological changes in placental tissue have been linked to chorangiosis, an adverse condition of the placenta itself, which is an indirect consequence for disease development and known to be associated with perinatal mortality and morbidity. Maternal exposure to urban ambient air pollution during the gestational period has been shown to lead to a significantly higher number of chorionic villi without a change in placental weight suggesting an increased possibility for developing

chorangiosis.[371] The associations between prenatal exposure to air pollution, the molecular changes in the placenta, and the consequences on developmental or disease characteristics later in life have not yet been studied extensively. Ghosh *et al.* investigated the effect of maternal gestational exposure to air pollution in relation to a specific placental genotype and the development of childhood bronchitis during the first two years of life.[49] A significant relationship was identified between the development of childhood bronchitis and the presence of a low activity EPHX1 polymorphism in the placenta with increased exposure to PAH and $PM_{2.5}$. These authors were thereby the first to identify a link between prenatal exposure to air pollution, a placental -omics marker and disease development.

Several studies have focused on placental -omics signatures and adverse birth outcomes such as reduced birth weight, intrauterine growth restriction, small for gestational age, and prematurity. In earlier studies, no significant associations were found between ambient air pollution exposure, birth weight and either the levels of DNA adducts in the placenta [56] or the activity of aryl hydrocarbon hydroxylase in placenta (AHH) [67], although a link was shown between air pollution exposure and AHH activity (see proteomics section). Three more recent studies found significant negative correlations between birth weight and the levels of NO_2 [48], $PM_{2.5}$ and PAHs [55], and the residential proximity to a major road.[60] However, Rossner *et al.* concluded that the levels of 8-oxo-deoxyguanosine (8-oxodG) DNA adducts, a marker of direct oxidative DNA damage, measured in placental tissue were only correlated with $PM_{2.5}$ levels but not with birth weight.[55] The same conclusion was reached for the association between residential distance to a major road and LINE element methylation: LINE methylation levels were only negatively associated with a distance to a major road, but no link with birth weight could be identified.[60] The only mediation analysis to investigate the triple association between air pollution exposure, placental –omics and disease development was performed with data of the INMA birth cohort.[48] This analysis showed that 10% of the association between a 10 $\mu g/m^3$ increase in NO_2 exposure during pregnancy and reduced birth weight could be mediated by a decrease in mitochondrial DNA levels. The results of these three studies could suggest that prenatal exposure to air pollution might exert its effects on birth outcomes by altering more subtle regulations such as those of the placental energy system and

not by direct damage to the placental DNA. However, since a broad array of possible molecular factors could be responsible for the link between *in utero* air pollution exposure and an effect on disease development later in life, integration of results on all -omics fields and the potential associations with prenatal exposure to ambient air pollution and childhood development should be prioritized in future research. This could eventually aid in the understanding of the complex etiology of adult diseases.

The strength of this review is that this is, to our knowledge, the first descriptive work to summarize and discuss the current knowledge on all placental -omics signatures that have been analyzed in association with prenatal air pollution exposure. This article has identified the current areas with the greatest gaps of knowledge which need to be addressed in future research and can therefore be a base to guide placental -omics research. A limitation in this review is the heterogeneity of the study designs of the 25 discussed articles. Differences were apparent in the approaches used to obtain the –omics data, the placental sampling protocols, and in both exposure assessment as well as the definition of the specific exposure windows. For these reasons a formal meta-analysis to combine the study results was not possible.

Table 6. Studies describing the triple relationship between exposure to ambient air pollution during pregnancy, the associated placental -omics marker and the health outcome.

Author	Exposure measured in ambient air	Placental measurement	Disease / health condition
Hincal F. (1986)	Urban air pollution	Placental AHH activity	Low birth weight and shorter birth length
Sram *et al.* (2006)	$PM_{2.5}$, PM_{10}	DNA adducts and *GSTM1, GSTP1, GSTT1, CYP1A1*2A* and *CYP1A1*2C* genotypes	Low birth weight and prematurity
Rossner *et al.* (2011)	PAHs, $PM_{2.5}$	SNP analysis for 95 genes and measurement of 8-oxodG adducts	Low birth weight and intrauterine growth restriction
Ghosh *et al.* (2013)	PAH, $PM_{2.5}$	Six SNPs (*GSTM1, GSTP1, GSTT1, CYP1A1 MspI, EPHX1* exon 3 and 4) and one *EPHX1* diplotype	Acute bronchitis in early childhood
Clemente *et al.* (2016)	NO_2	mtDNA content	Low birth weight
Kingsley *et al.* (2016)	Residential proximity to a major road	DNA methylation (LINE and AluYb8 elements)	Low birth weight and small for gestational age

Abbreviations: 8-oxodG, (8-oxo-2'-deoxyguanosine); AHH, Aryl hydrocarbon hydroxylase; CYP1A1(*2A), Cytochrome (CYP) P450 1A1 (2A); EPHX1, Epoxide hydrolase 1; GSTM1, Glutathione S-transferase M1; GSTP1, Glutathione S-transferase P1; GSTT1, Glutathione S-transferase T1; His, Histidine; LEP, Leptin; miR, MicroRNA; mtDNA, Mitochondrial DNA; MspI, Substitution of isoleucine to valine in the 3' non-coding region of CYP1A1, NO_2, Nitrogen dioxide; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; PM_{10}, Particulate matter with a diameter smaller than 10 μm; SNP, Single nucleotide polymorphism.

APPENDIX A

CONCLUSIONS AND FUTURE DIRECTIONS

Exposure to air pollution in daily life is unavoidable. A crucial time window of exposure in the course of human life is fetal development. The feto-placental unit is subjected to maternal conditions and exposures that can adversely affect -omics characteristics of the placenta. Eventually, these placental changes could potentially lead to alterations in metabolic capacities of the fetus and an increased risk of disease development later in life. This systematic review shows that the placenta is a suitable tissue to investigate the effects of prenatal exposure to ambient air pollution by examining -omics biomarkers. The placenta is a temporary organ that reflects various exposures throughout pregnancy. Important in this branch of research is to have a representation of the effects of these exposures on the whole placenta. Since not only inter-, but also intra-placental differences should always be taken into account, researchers should try to find a consensus on a unified, standardized method to work with a pooled sample of each placenta. Especially in -omics research, placental sampling is crucial because of the fragility of DNA, protein and especially RNA structures. Therefore, standardization and communication about sampling methods is crucial in -omics research. In this way, results over different cohorts could be more easily compared and discussed.

At this point in time, this systematic review shows that some -omics fields are more represented than others in the research on the effects of prenatal exposure to air pollution on placental biomarkers. The most focus has been put on the presence of placental DNA adducts, although only a minority of studies found significant effects of air pollution exposure on these biomarkers. Therefore, more attention should be put on other, more promising -omics fields such as epigenetics, transcriptomics, and proteomics. At present, several placental -omics markers have been suggested that could provide a better insight on how the consequences of exposure to ambient air pollution are manifested during pregnancy. However, most studies only focus on specific components of molecular systems and pathways. Integrating a top-down approach is crucial for epigenomics, transcriptomics and proteomics for a full understanding of the array of molecular changes that result from detrimental environmental exposures. More studies containing large qualitative datasets should combine candidate -omics

markers with the exploration of entire metabolic pathways in the full genome, transcriptome, epigenome, proteome or metabolome. Eventually, this should provide a complete molecular signature of key players describing the effects of prenatal environmental exposure on placental functioning and fetal (disease) development.

Two -omics fields, - metabolomics and exposomics -, could not be sufficiently covered in the context of this systematic review because of the current paucity of such studies. More attention should be put on these fields to further expand the knowledge on placental biomolecular signatures of prenatal air pollution exposure. In general, the effects of detrimental exposures on placental molecular changes and the subsequent effects on the programming of pathologies later in life are rather scarcely documented. Therefore, future research should focus more on integrative projects such as the epigenome-wide association studies (EWAS) to identify key molecular regulators in the etiology of disease processes. Also, more longitudinal follow-up research is needed to identify and clarify the triple link between *in utero* exposure to ambient air pollution, changes in placental -omics categories, and disease initiation/progression later in life. Two projects that are already integrating several hazardous exposures such as ambient air pollution, the molecular signatures of these exposures in several tissues and the health effects on newborns, children and adults are the Human Early-Life Exposome (HELIX) project [372] and the EXPOsOMICS project.[373] In conclusion, future integrative long-term research looks promising in elucidating the underlying placental mechanisms that potentially influence disease development later in life, as a consequence of gestational air pollution exposure.

APPENDIX A

SUPPLEMENTAL MATERIAL

Supplemental Table S1: List of MeSH-terms used in the search of articles for this systematic review.

Search term category	MeSH-terms inserted in Pubmed
Terms related to placenta	- placenta - placental development - placental transfer
Terms related to air pollution exposure	- air pollution - ambient air pollution - air pollution health - particulate matter - $PM_{2.5}$ - $PM_{2.5}$ PM_{10} - particulate matter 2.5 - particulate matter 10 - ultrafine particles - black carbon - NOx - NO_2 - PAH - polycyclic aromatic hydrocarbon air
Terms related to disease development in early life	- fetal origin adult disease - barker hypothesis - barker hypothesis fetal - barker hypothesis fetal origins

Abbreviations: NO_2, Nitrogen dioxide; NOx, Nitrogen oxides; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; PM_{10}, Particulate matter with a diameter smaller than 10 μm.

Supplemental Table S2. List of search strings entered in the Pubmed search engine and the corresponding number of hits.

Search string in Pubmed	Number of hits
placenta AND air pollution	118
placenta AND ambient air pollution	9
placenta AND air pollution health	43
placenta AND particulate matter	112
placenta AND PM2 5	6
placenta AND PM2 5 PM10	0
placenta AND particulate matter 2 5	20
placenta AND particulate matter 10	36
placenta AND ultrafine particles	2
placenta AND black carbon	2
placenta AND NOx	16
placenta AND NO2	15
placenta AND PAH	66
placenta AND polycyclic aromatic hydrocarbons air	21
placenta AND barker hypothesis	12
placenta AND barker hypothesis fetal	7
placenta AND barker hypothesis fetal origins	5
placenta AND fetal origin adult disease	58
placenta development AND air pollution	35
placenta development AND ambient air pollution	8
placenta development AND air pollution health	17
placenta development AND particulate matter	23
placenta development AND PM2 5	1
placenta development AND PM2 5 PM10	0
placenta development AND particulate matter 2 5	6
placenta development AND particulate matter 10	9
placenta development AND ultrafine particles	2
placenta development AND black carbon	5
placenta development AND NOx	19
placenta development AND NO2	19
placenta development AND PAH	16
placenta development AND polycyclic aromatic hydrocarbons air	17

Continued

Supplemental Table S2. Continued

Search string in Pubmed	Number of hits
placenta development AND barker hypothesis	16
placenta development AND barker hypothesis fetal	15
placenta development AND barker hypothesis fetal origins	7
placenta development AND fetal origin adult disease	17
placenta transfer AND air pollution	3
placenta transfer AND ambient air pollution	0
placenta transfer AND air pollution health	2
placenta transfer AND particulate matter	2
placenta transfer AND PM2 5	1
placenta transfer AND PM2 5 PM10	0
placenta transfer AND particulate matter 2 5	0
placenta transfer AND particulate matter 10	0
placenta transfer AND ultrafine particles	1
placenta transfer AND black carbon	0
placenta transfer AND NOx	0
placenta transfer AND NO2	0
placenta transfer AND PAH	0
placenta transfer AND polycyclic aromatic hydrocarbons air	0
placenta transfer AND barker hypothesis	0
placenta transfer AND barker hypothesis fetal	0
placenta transfer AND barker hypothesis fetal origins	0
placenta transfer AND fetal origin adult disease	0
barker hypothesis AND air pollution	2
barker hypothesis AND ambient air pollution	0
barker hypothesis AND air pollution health	0
barker hypothesis AND particulate matter	3
barker hypothesis AND PM2 5	0
barker hypothesis AND PM2 5 PM10	0
barker hypothesis AND particulate matter 2 5	0
barker hypothesis AND particulate matter 10	0
barker hypothesis AND ultrafine particles	0
barker hypothesis AND black carbon	0
barker hypothesis AND NOx	0

Continued

Supplemental Table S2. Continued

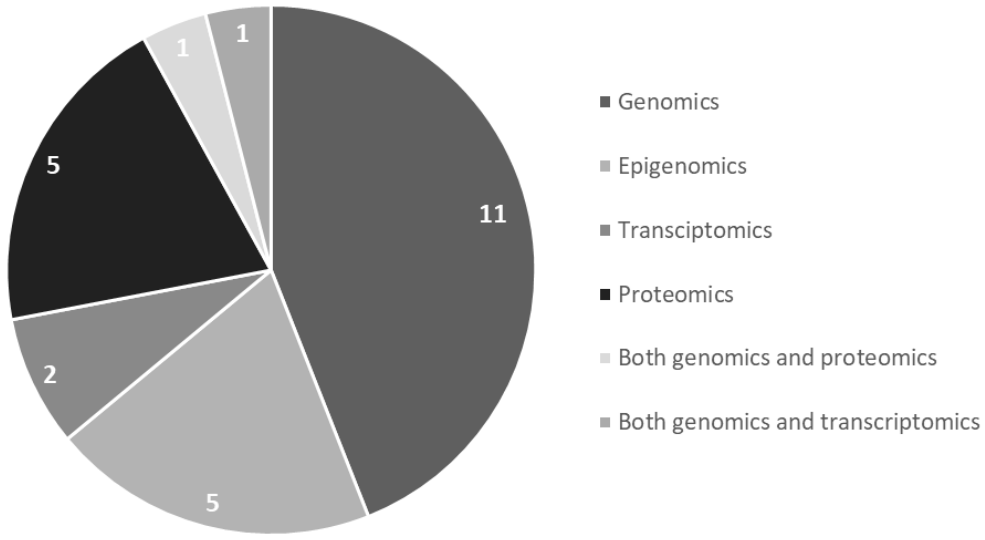
Search string in Pubmed	Number of hits
barker hypothesis AND NO2	0
barker hypothesis AND PAH	0
barker hypothesis AND polycyclic aromatic hydrocarbons	0
fetal origin adult disease AND air pollution	2
fetal origin adult disease AND ambient air pollution	0
fetal origin adult disease AND air pollution health	0
fetal origin adult disease AND particulate matter	0
fetal origin adult disease AND PM2 5	0
fetal origin adult disease AND PM2 5 PM10	0
fetal origin adult disease AND particulate matter 2 5	0
fetal origin adult disease AND particulate matter 10	0
fetal origin adult disease AND ultrafine particles	0
fetal origin adult disease AND Polycyclic aromatic hydrocarbons	0
fetal origin adult disease AND PAH	1
fetal origin adult disease AND black carbon	0
fetal origin adult disease AND NOx	0
fetal origin adult disease AND NO2	0
barker hypothesis fetal AND air pollution	1
barker hypothesis fetal AND ambient air pollution	0
barker hypothesis fetal AND air pollution health	0
barker hypothesis fetal AND particulate matter	0
barker hypothesis fetal AND PM2 5	0
barker hypothesis fetal AND PM2 5 PM10	0
barker hypothesis fetal AND particulate matter 2 5	0
barker hypothesis fetal AND particulate matter 10	0
barker hypothesis fetal AND ultrafine particles	0
barker hypothesis fetal AND Polycyclic aromatic hydrocarbons	0
barker hypothesis fetal AND PAH	0
barker hypothesis fetal AND black carbon	0
barker hypothesis fetal AND NOx	0
barker hypothesis fetal AND NO2	0
barker hypothesis fetal origins AND air pollution	1
barker hypothesis fetal origins AND ambient air pollution	0

Continued

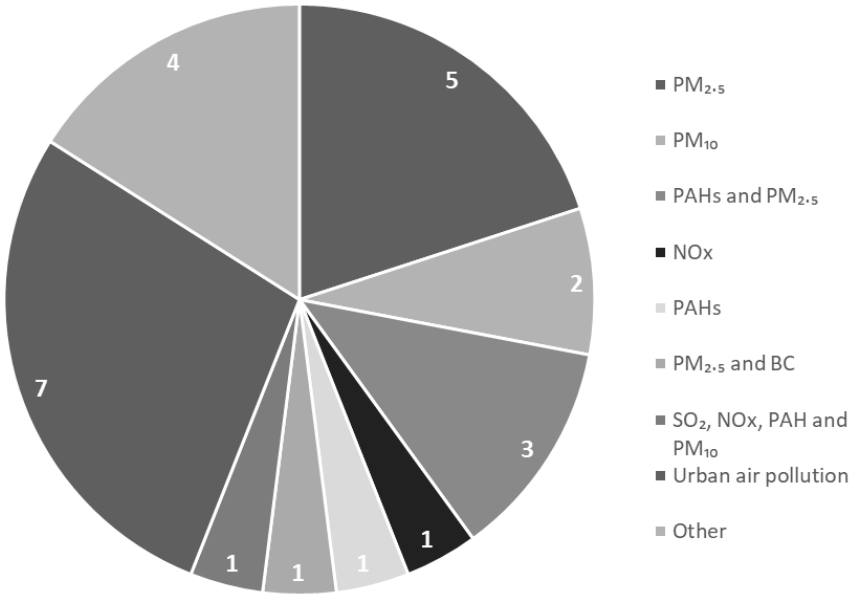
Supplemental Table S2. Continued

Search string in Pubmed	Number of hits
barker hypothesis fetal origins AND air pollution health	0
barker hypothesis fetal origins AND particulate matter	0
barker hypothesis fetal origins AND PM2 5	0
barker hypothesis fetal origins AND PM2 5 PM10	0
barker hypothesis fetal origins AND particulate matter 2 5	0
barker hypothesis fetal origins AND particulate matter 10	0
barker hypothesis fetal origins AND ultrafine particles	0
barker hypothesis fetal origins AND Polycyclic aromatic hydrocarbons	0
barker hypothesis fetal origins AND PAH	0
barker hypothesis fetal origins AND black carbon	0
barker hypothesis fetal origins AND NOx	0
barker hypothesis fetal origins AND NO2	0

Abbreviations: NO_2, Nitrogen dioxide; NOx, Nitrogen oxides; PAH, Polycyclic aromatic hydrocarbon; $PM_{2.5}$, Particulate matter with a diameter smaller than 2.5 μm; PM_{10}, Particulate matter with a diameter smaller than 10 μm.



<u>Supplemental Figure 1.</u> Pie chart showing the fields of placental -omics biomarkers that are represented in this systematic review, and the respective number of discussed articles that can be appointed to each -omics field.



<u>Supplemental Figure 2.</u> Pie chart showing the forms of prenatal air pollution exposure that were investigated in the articles of this systematic review, and the respective number of discussed articles that can be appointed to each category. The category 'Other' includes the distance of the residence to a major road, residential traffic density, work-related air pollution exposure in Mexican factories, and smoke from residential coal burning as a heating source.

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CURRICULUM VITAE

Leen Luyten was born in Genk on the 1st of February 1992. After she graduated from secondary school in 2010, the started her bachelor study in Biomedical Sciences at Hasselt University and graduated her master's degree in Environmental Health Sciences in 2015, *magna cum laude*. In the same year, she started her joined PhD in molecular epidemiology at both the Centre for Environmental Sciences at Hasselt University and at the Unité de Recherche en Biologie Cellulaire (URBC) – Namur Research Institute for Life Sciences (Narilis) at Namur University. Her PhD project was the first to be engaged between the two universities via a Bijzonder Onderzoeksfond (BOF) grant, which was officially inaugurated by the governors of the province of Limburg and the province of Namur. During her PhD, Leen focussed on unravelling the effects of air pollution exposure during pregnancy on the placental proteome, and on the microvascular and neurocognitive test outcomes of children at the age of 4. For the latter, she went to Cambridge to the discuss the neurocognitive test outcomes with the developing team of the CANTAB test battery. Besides teaching activities, she performed many clinical visits of the ENVIR*ON*AGE follow-up cohort. She presented the results of het PhD at several national and international conferences, such as the BePA conference in Gent (Belgium), the ISEE conferences in Rome (Italy) and Utrecht (The Netherlands) and the DOHaD conference in Rotterdam (The Netherlands).

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2. **Luyten LJ**, Saenen ND, Janssen BG, Vrijens K, Plusquin M, Roels HA, Debacq-Chainiaux F, Nawrot TS. Air pollution and the fetal origin of disease: A systematic review of the molecular signatures of air pollution exposure in human placenta. Environ Res. 2018;166(March):310–23.

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9. **Luyten LJ**, Dieu M, Bové H, Demazy C, Fransolet M, Renard P, Nawrot TS, Debacq-Chainiaux F. Prenatal black carbon exposure influences the placental proteome. *In preparation.*

ABSTRACTS

1. **Luyten LJ**, Vrijens K, Saenen ND, Roels HA, Debacq-Chainiaux F, Nawrot TS. Placental TRPC6 expression and gestational trimester-specific $PM_{2.5}$ air pollution exposure in the ENVIR*ON*AGE birth cohort. 28 th Conference of the International Society for Environmental Epidemiology, Rome, Italy, 1-4 September 2016 (**oral presentation**).

2. **Luyten LJ**, Dieu M, Renard P, Nawrot TS, Debacq-Chainiaux F. Optimization of label-free MS techniques for the identification and quantification of key placental proteins in association with exposure to PM air pollution. Belgian Proteomics Association (BePA), Gent, Belgium, 17-18 October 2016 (**poster presentation**).

3. **Luyten LJ**, Fransolet M, Karen V, Tsamou M, Saenen ND, Wang C, Nawrot TS, Debacq-Chainiaux F. Placental canonical TRP channel 6 expression and gestational trimester-specific fine particle air pollution exposure in the ENVIR*ON*AGE birth cohort. 10th World Congress on Developmental origins of health and disease: Life course health & disease: Observations, experiments and interventions, Rotterdam, The Netherlands, 15-18 October 2017, (**oral presentation**).

4. **Luyten LJ**, Dockx Y, Provost EB, Madhloum N, Sleurs H, Neven KY, Janssen BG, Bové H, Debacq-Chainiaux F, Gerrits N, Lefebvre W, Plusquin M, Vanpoucke C, De Boever P, Nawrot TS. Children's microvascular traits and air pollution exposure during pregnancy and early childhood: prospective evidence to elucidate the developmental origin of particle-induced disease. 31st Annual Conference of the International Society for Environmental Epidemiology, Utrecht, The Netherlands 25-28 August 2019 (**poster presentation**).

DANKWOORD

DANKWOORD

After reading so many thesis acknowledgements, it's finally time to write mine. Thank you for reading this thesis and my papers (even if you skipped right to this section, I would have probably done the same thing) and I hope you had as much joy in reading it as I had in writing it. During the past 4 years as PhD candidate (and a year extra to write my thesis booklet and papers during the next step of my professional career) I have learned so many new things and had so many amazing experiences, which I couldn't have had without all the people I have met during this PhD as well as all the people who have been there for me before and after this period in my life. To thank all these people, because of the multilingual character of my PhD, I will switch a couple of times in the sections below between Dutch, English and French (or at least I will try my best for the latter).

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